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Pheromone-Dependent Destruction of the Tec1 Transcription Factor Is Required for MAP Kinase Signaling Specificity in Yeast

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Summary

The yeast MAPK pathways required for mating versus filamentous growth share multiple components yet specify distinct programs. The mating-specific MAPK, Fus3, prevents crosstalk between the two pathways by unknown mechanisms. Here we show that pheromone signaling induces Fus3-dependent degradation of Tec1, the transcription factor specific to the filamentation pathway. Degradation requires Fus3 kinase activity and a MAPK phosphorylation site in Tec1 at threonine 273. Fus3 associates with Tec1 in unstimulated cells, and active Fus3 phosphorylates Tec1 on T273 in vitro. Destruction of Tec1 requires the F box protein Dia2 (*D*igs-*i*nto-*a*gar-*2*), and Cdc53, the Cullin of SCF (*S*kp1-*C*dc53-*F* box) ubiquitin ligases. Notably, mutation of the phosphoacceptor site in Tec1, deletion of *FUS3,* or deletion of *DIA2* results in a loss of signaling specificity such that pheromone pathway signaling erroneously activates filamentation pathway gene expression and invasive growth. Signal-induced destruction of a transcription factor for a competing pathway provides a mechanism for signaling specificity.

Introduction

Eukaryotic intracellular signaling systems often share components. A central question in cell biology is how these systems transduce different extracellular signals to produce appropriate responses. In the budding yeast *Saccharomyces cerevisiae*, the mitogen-activated protein kinase (MAPK) pathways that mediate the mating pheromone response and filamentous growth share multiple cascade components, including the Ste20 PAK, the Ste11 MEKK, and the Ste7 MEK (Figure 1) (Dohlman and Thorner, 2001; Madhani and Fink, 1998; Schwartz and Madhani, 2004). However, each pathway activates a distinct transcriptional program through different ERK-type MAPKs: Fus3 for the pheromone response and Kss1 for the filamentation response. Pheromone-responsive transcription is mediated by the transcription factor Ste12, which functions as a homomultimer or as a heteromultimer with the Mcm1 MADS box protein (Dohlman and Thorner, 2001). Filamentation-specific gene expression is activated by the TEA/ATTS family transcription factor Tec1 forms a heteromultimer with Ste12 (Baur et al., 1997; Gavrias et al., 1996; Madhani and Fink, 1997; Mosch and Fink, 1997). Purified recombinant Tec1 and Ste12 bind cooperatively in vitro to filamentation response elements (FREs), which are sufficient to program gene expression that is dependent on the filamentation MAPK pathway (Madhani and Fink, 1997; Mosch et al., 1996).

Pheromone treatment induces phosphorylation and activation of Fus3 (Elion et al., 1993). Surprisingly, a fraction of Kss1 molecules is also phosphorylated in response to mating pheromone (Breitkreutz et al., 2001; Cherkasova et al., 2003; Sabbagh et al., 2001). Although the mechanism of this activation is unknown, phosphorylation of Kss1 by mating pheromone does not induce FRE-dependent gene expression in wild-type cells of the filamentation-competent laboratory strain Σ1278b, suggesting the existence of a suppressive mechanism that acts downstream of Kss1 phosphorylation to prevent crosstalk (Madhani et al., 1997).

Although virtually nothing is known about the mechanisms that maintain signaling specificity during the mating versus invasive growth responses in yeast, one clue is provided by the observation that the mating pathway-specific MAPK Fus3 is required for signaling specificity. In cells lacking Fus3 or Fus3 kinase activity, pheromone signaling results in increased phosphorylation of Kss1, activation of filamentation pathway-specific gene expression (as assayed by *FRE-lacZ* reporter and DNA microarrays), and a relocalization of Ste12 from mating promoters to filamentation gene promoters (Madhani et al., 1997; Roberts et al., 2000; Sabbagh et al., 2001; Zeitlinger et al., 2003). However, the targets of the Fus3 MAPK required for specificity have not been described.

Here, we describe a key mechanism by which Fus3 promotes signaling specificity. We show that Fus3 binds to and phosphorylates Tec1, which in turn induces its degradation. We show that Tec1 degradation requires a novel SCF ubiquitin ligase containing the Dia2 F box specificity subunit. Mutations in *FUS3*, *TEC1*, or *DIA2* that block Tec1 degradation produce a loss of signaling specificity such that the pheromone signal now activates filamentation pathway-specific gene expression and invasive growth.

Results and Discussion

We examined the effects of α-pheromone treatment of haploid mating type **a** cells on the levels of a functional myc epitope-tagged version of Tec1 expressed from its natural promoter on a centromeric plasmid. Tec1 levels declined rapidly within 5 min of pheromone treatment, consistent with rapid degradation (Figure 2A). Supporting the view that pheromone causes Tec1 degradation rather than blocking its synthesis, a recent genome-wide study demonstrated that pheromone treatment increases *TEC1* mRNA levels several-fold and has no ef-

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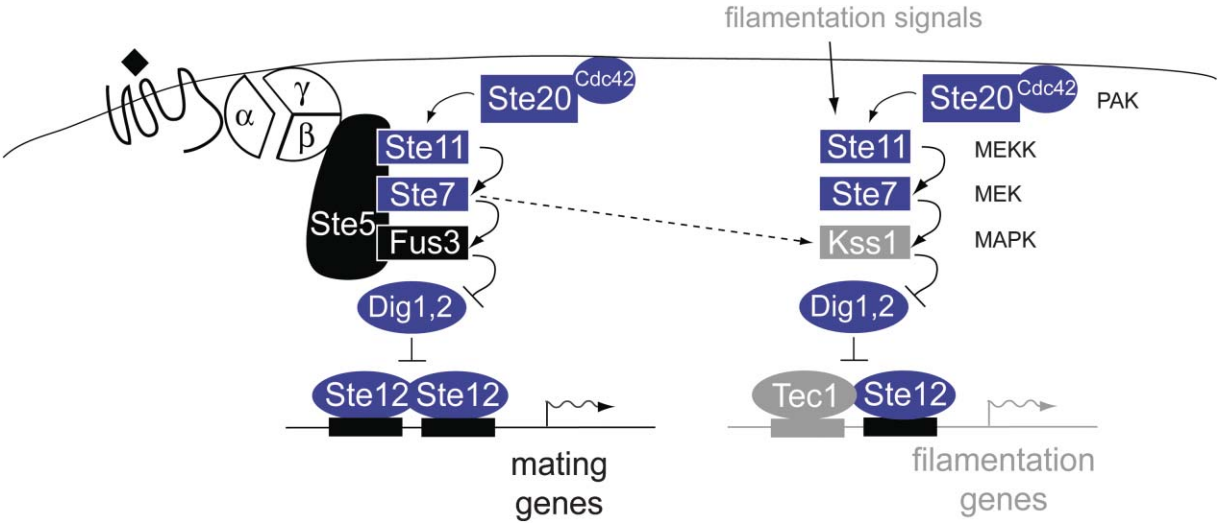

Figure 1. Diagram of the MAP Kinase Pathways that Control the Mating Pheromone Response and Filamentous Growth in *Saccharomyces cerevisiae*

Mating-specific components are depicted in black, filamentation-specific components in gray, and shared components in blue. PAK, p21-activated kinase; MEKK, MEK kinase; MEK, MAP/ERK kinase; MAPK, mitogen-activated protein kinase.

fect on translational efficiency of the *TEC1* mRNA as determined by ribosome density measurements (MacKay et al., 2004).

Since proteins are often degraded by the ubiquitin system, we determined if Tec1 forms ubiquitin conjugates in vivo. For these experiments, we expressed a His6-tagged proteolysis-resistant mutant form of ubiquitin (*His6-myc-Ub-K48R, G76A*) in cells harboring a FLAG epitope-tagged version of Tec1 as the sole source of Tec1 (Finley et al., 1994). We performed nickel-NTA affinity selections under denaturing conditions in extracts from cells harboring either the His6-tagged mutant ubiquitin or an untagged mutant ubiquitin. Analysis of selected material by SDS-PAGE and immunoblotting with anti-FLAG antibodies revealed a ladder of bands with a lower mobility that that of FLAG-Tec1, indicating ubiquitin conjugates (Figure 2B). These bands were absent in material selected from cells harboring the untagged form *ubi-K48R, G76A* allele, demonstrating the specificity of the affinity selection for the His6-tagged ubiquitin (Figure 2B). Moreover, as for degradation, pheromone treatment was required for the detection of robust ubiquitin conjugates (Figure 2B).

To determine if Tec1 degradation required the Fus3 MAPK, time course experiments were repeated in *fus3*Δ strains and strains expressing a kinase-dead allele, *fus3-K42R*. In these strains, pheromone had no effect on myc-Tec1 levels (Figures 3A and 3B), consistent with a model in which phosphorylation of Tec1 by Fus3 induces its degradation. In contrast, Tec1 destruction in response to pheromone was normal in cells lacking the filamentation MAPK Kss1 (Figure 3C).

Among a set of previously isolated hyperinvasive alleles of *TEC1*, two affected the residues T273 and P274 (Kohler et al., 2002) that together form a consensus phosphorylation site for proline-directed kinases such as MAPKs (Chen et al., 2001). Consistent with the model that Fus3 phosphorylates T273 to promote Tec1 degradation, we found that the myc-Tec1-T273M and -P274S mutants were completely resistant to pheromone-induced degradation in vivo (Figures 3D and 3E).

To determine if Fus3 associates with Tec1 in vivo, we performed coimmunoprecipitation experiments using extracts from cells expressing a chromosomal *FUS3-FLAG* epitope-tagged allele and plasmid-borne *myc-TEC1* allele. Immunoprecipitation of Fus3-FLAG resulted in the efficient coenrichment of myc-Tec1 (Figure 4A). To determine whether Fus3 could phosphorylate Tec1, we immunoprecipitated Fus3-FLAG or Fus3-K42R-FLAG from cells that were untreated or treated with mating pheromone for 1 hr. The immunoprecipitates were incubated with purified recombinant full-length MBP-Tec1-FLAG in the presence of [γ-^{32}P]ATP. MBP-Tec1-FLAG was phosphorylated when incubated with Fus3-FLAG immunopurified from pheromone-treated cells but not using Fus3-FLAG immunopurified from unstimulated cells (Figure 4B). In contrast, Fus3-K42R-FLAG immunoprecipitates did not harbor activity toward the recombinant Tec1 substrate, and reactions lacking MBP-Tec1-FLAG did not display a labeled band (Figure 4B). To determine what sites were phosphorylated by Fus3, we performed LC/MS/MS mass spectrometry on trypic digests of MBP-Tec1-FLAG phosphorylated in vitro (see Experimental Procedures). This analysis covered 39.5% of the protein sequence of Tec1 and demonstrated phosphorylation on T273 as well as phosphorylation of four neighboring residues (S269, T276, T289, T297). In addition, phosphorylation was detected of a second cluster of residues (S399, T401) as well as of S421 (Figure 4C). Figure 4D shows an MS/MS spectrum corresponding to fragment ions derived from a peptide parent ion dually phosphorylated at T273 and T276. The requirement for a consensus MAPK site corresponding to T273 on Tec1 and Fus3 kinase activity for Tec1 destruction in vivo, the association of Fus3 with Tec1 in unstimulated cells, and the ability of Fus3 to

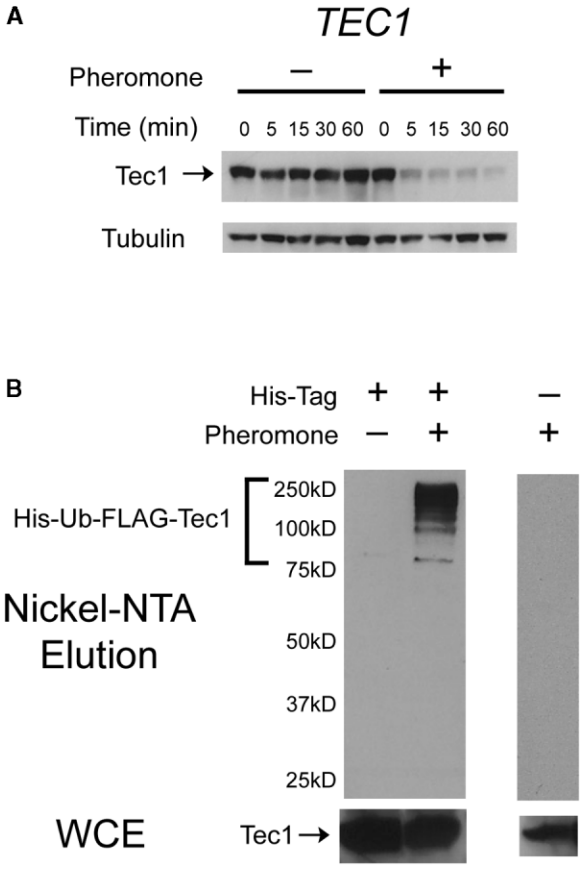

Figure 2. Pheromone Signaling Induces Rapid Loss of Tec1 and the Formation of Tec1 Ubiquitin Conjugates

(A) Time course experiments. Immunoblot of extracts from *tec1*Δ cells of the Σ1278b strain background harboring *myc-TEC1* expressed from its natural promoter on a *CEN* plasmid grown at 30°C and either treated or not with α-pheromone for the indicated times. Blots were probed with anti-myc to visualize myc-Tec1 and reprobed with an anti-α-tubulin antibody as a loading control.

(B) Ubiquitin conjugates. Immunoblot using anti-FLAG antibodies of ubiquitin conjugates isolated by Nickel-NTA affinity chromatography under denaturing conditions from extracts of cells expressing His6-Ub-K48R,G76A and FLAG-Tec1 and treated or not with pheromone. WCE panels (bottom) are from separate exposures of two blots.

phosphorylate recombinant Tec1 in vitro on T273 support the model that phosphorylation of T273 by Fus3 initiates Tec1 destruction.

In organisms ranging from yeast to humans, the degradation of phosphorylated proteins often depends on SCF (*S*kp-*C*ullin-*F* box) E3 ubiquitin ligases (Deshaies, 1999; Patton et al., 1998; Pintard et al., 2004). These protein complexes contain a common Cullin subunit and distinct F box proteins that directly recognize specific phosphorylated substrates. F box proteins have one of two substrate recognition domains: WD-40 repeats or leucine-rich repeats (LRRs). The yeast genome encodes at least 17 F box proteins (Patton et al., 1998). One of these proteins, Dia2 (*D*igs *I*nto *A*gar *2*), is a member of the LRR class. *DIA2* was first identified in a genetic screen as a repressor of invasive growth (Palecek et al., 2000). A recent proteomics study of yeast F box proteins demonstrated bona fide ubiquitin ligase activity for a purified reconstituted SCF^{Dia2} complex (Kus et al., 2004). The hyperinvasive phenotype of *dia2*Δ cells is consistent with that expected for an F box protein required for SCF-dependent destruction of Tec1.

Notably, we found that myc-Tec1 levels were unaffected in pheromone-treated cells lacking Dia2 (Figure 5A). Thus, Tec1 degradation in response to pheromone signaling requires a specific SCF-associated F box protein.

To rule out the possibility that *dia2*Δ mutant cells fail to degrade Tec1 in response to pheromone because of a defect in the activation of Fus3 by phosphorylation, we monitored the phosphorylation of the activation loops of the Fus3 and Kss1 MAPKs in response to pheromone treatment of cells using an anti-ERK2 phosphospecific antibody that crossreacts with activated forms of Fus3 and Kss1 (Sabbagh et al., 2001). We found no effect of *dia2*Δ on pheromone-induced phosphorylation of these MAPKs either in cells lacking (Figure 5B) or containing *TEC1* (data not shown).

The Cullin subunit of yeast SCF is Cdc53, and its principal E2 is Cdc34. To determine whether these components were required for degradation of myc-Tec1, we analyzed the pheromone response in *cdc53-1*^{ts} and *cdc34-2*^{ts} cells at nonpermissive temperature (Figures 5C, 5D, and 5E). Pheromone-dependent destruction of myc-Tec1 was abolished in these strains but not in the isogenic wild-type control strain. In this filamentation-defective strain background (W303), degradation of myc-Tec1 in the wild-type strain is considerably slower than in the filamentation-competent Σ1278b strains used the experiments described above. Nonetheless, we observed a consistent degradation defect in the mutants.

Taken together, our data show that pheromone-induced degradation of Tec1 requires a specific ubiquitin ligase, SCF^{Dia2}. Since SCF E3s from yeast, plants, and mammals specifically bind their phosphorylated substrates via F box proteins, it seems likely that SCF^{Dia2} directly recognizes phosphorylated Tec1 to induce its ubiquitination and destruction. However, further work will be necessary to rule out the possibility that Dia2 acts indirectly and/or in concert with other factors to promote the degradation of Tec1.

We next examined the role of Tec1 destruction in signaling specificity. As described in the Introduction, cells lacking the mating pathway MAPK Fus3 display a defect in signaling specificity: in the absence of mating pheromone, *fus3*Δ and *fus3-K42R* cells display increased FRE-dependent gene expression and invasive growth due to crosstalk from basal signaling through the pheromone response pathway. In response to pheromone treatment, this erroneous signaling to the filamentation pathway increases. To determine if phosphorylation of Tec1 is important for signaling specificity, we examined *FRE-lacZ* levels in cells harboring *tec1-T273M* and *tec1-P274S* alleles. As with a *fus3* mutant, these cells expressed increased *FRE-lacZ* compared to the wild-type control (Figure 6A). To determine whether increased *FRE-lacZ* expression was due to crosstalk from the mating MAPK pathway, we deleted the gene encoding the pheromone pathway-specific receptor-coupled Gβ protein, Ste4, which is essential for basal

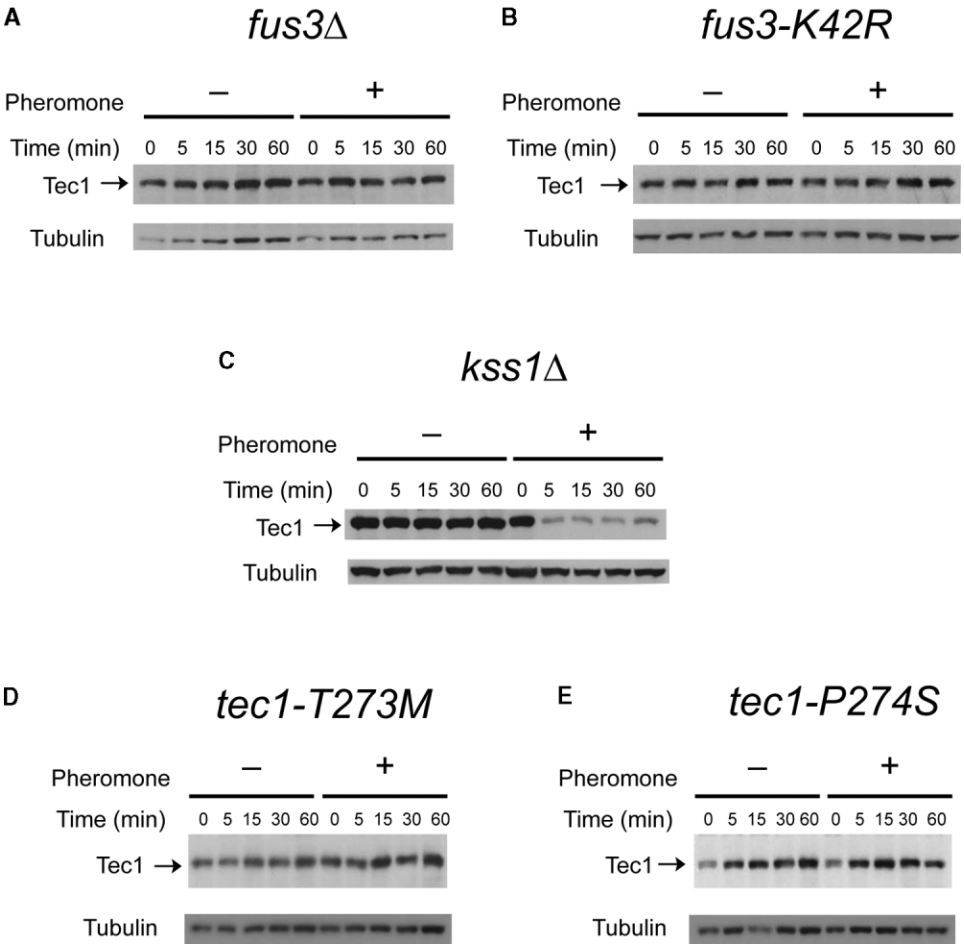

Figure 3. Genetic Requirements for Tec1 Degradation

(A–E) Time course experiments. Strains are of the Σ1278b background and carry a chromosomal deletion of *TEC1*, which was covered by *CEN* plasmid expressed wild-type myc-*TEC1* as in Figure 2A (A–C) or the indicated myc-tagged mutant *TEC1* allele (D, E). In (B), the strain is the same as that in (A) except a plasmid carrying the mutant *fus3-K42R* allele was also present. Strains were grown at 30°C and analyzed as in Figure 2A.

and pheromone-induced signaling through the mating pathway. Deletion of *STE4* reversed the hyperactivation of *FRE-lacZ* seen in the *TEC1* phosphoacceptor site mutants, demonstrating that these residues are necessary to prevent crosstalk, but not filamentation signaling (Figure 6A). In addition, upon pheromone treatment, a further increase in *FRE-lacZ* expression is seen in *tec1-T273M* and *tec1-P274S* mutant cells, but not in wild-type cells (Figure 6A).

We also determined the effects of the Tec1 site mutants on the activity of a filamentation gene promoter, that of the *PGU1* gene, which encodes a secreted pectinase (Madhani et al., 1999). We found that while deletion of *STE4* had no effect on the expression of *PGU1-lacZ*, the *tec1-T273M* and *tec1-P274S* mutants caused an increase in expression that was *STE4*-dependent (Figure 6B). Thus for both reporter genes, mutations in the phosphoacceptor site in Tec1 that block degradation also produce crosstalk.

Analysis of *FRE-lacZ* expression in the *fus3Δ tec1-T273M* double mutant revealed no difference with the *fus3Δ* single mutant, supporting the notion that Fus3 and T273 in Tec1 function in the same pathway to suppress crosstalk (Figure 6C). To further test the link between Tec1 degradation and crosstalk, we examined *FRE-lacZ* expression in *dia2Δ* cells. We found that *FRE-lacZ* expression in *dia2Δ* cells was *STE4*-dependent in the absence of pheromone and induced by pheromone treatment of cells, further supporting the model that degradation is necessary to suppress crosstalk (Figure 6D). The basal expression levels of *FRE-lacZ* was lower in *dia2Δ* cells compared to wild-type, suggesting additional roles for Dia2 the filamentation pathway.

The *tec1-T273M* and *tec1-P274S* alleles were isolated previously as hyperinvasive mutants (Kohler et al., 2002). To determine whether this phenotype was due to a loss of signaling specificity, we determined whether the hyperinvasive phenotype required *STE4*. Comparison of the invasive growth phenotypes of the *tec1-T273M* and *tec1-P274S* alleles in the presence and absence of *STE4* revealed that the hyperinvasive phenotype of the *TEC1* phosphoacceptor site mutants requires pheromone pathway signaling (Figure 6E).

Our results demonstrate an inhibitory mechanism by

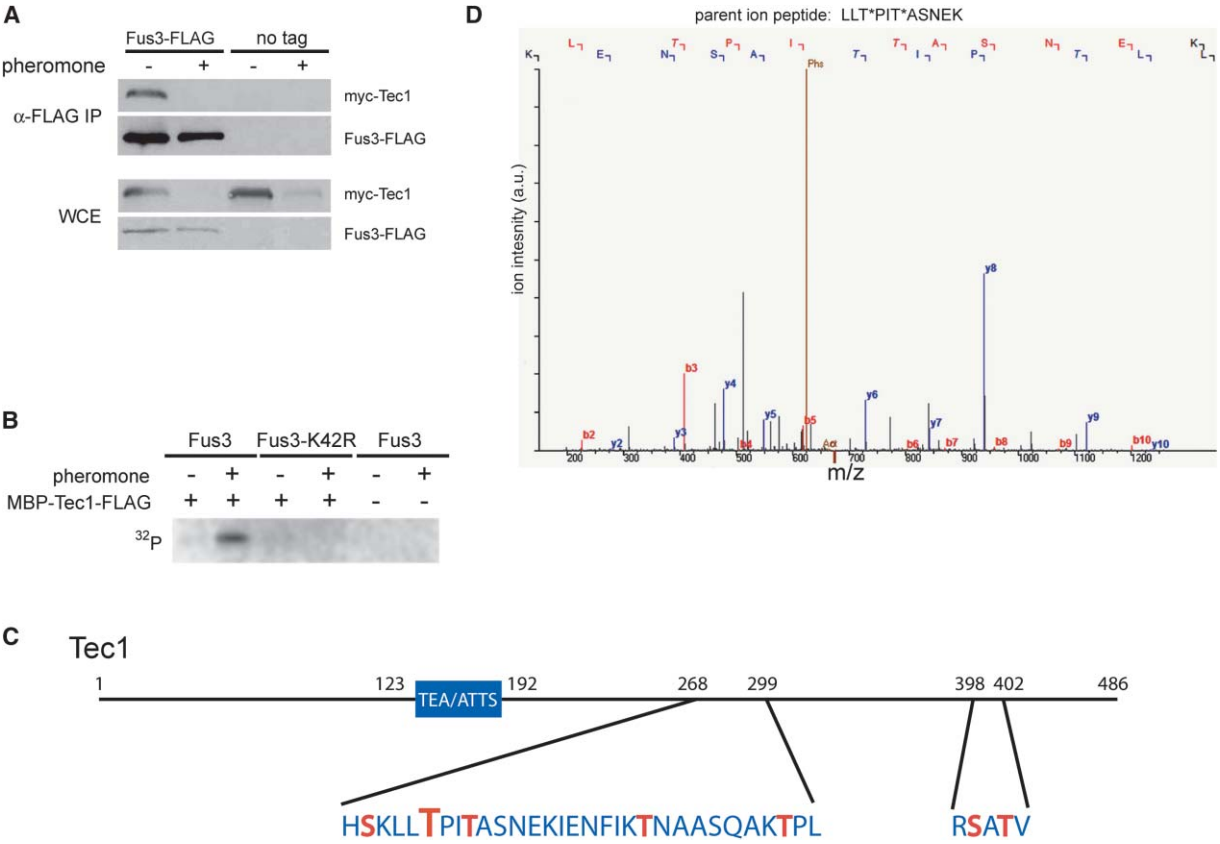

Figure 4. Fus3 MAPK Associates with and Phosphorylates Tec1

(A) Coimmunoprecipitation of Tec1 with Fus3. A stable complex between myc-Tec1 and Fus3-FLAG is formed. Immunoprecipitates and 25μg of whole-cell extracts from the indicated strains were analyzed by immunoblotting with anti-myc or anti-FLAG antibody as indicated.

(B) Kinase assays. Autoradiogram showing the products of in vitro kinase assays using [γ-^{32}P]ATP. Immunopurified FLAG-tagged, endogenously expressed MAPKs from the indicated strains were incubated with recombinant MBP-Tec1-FLAG substrate. Shown in a 2 hr exposure.

(C) Phosphorylation sites of Tec1 identified by mass spectrometry. Shown is a linear depiction of the Tec1 protein sequence and the position of its TEA/ATTS DNA binding domain. The two major clusters of in vitro phosphorylation sites indicated. Phosphorylated sites are depicted in red. Threonine 273 is shown in a larger font for emphasis.

(D) Spectrum. Shown is the experimentally collected MS/MS spectra generated from collision induced dissociation and subsequently matched by SEQUEST to the doubly phosphorylated peptide LLT*PIT*ASNEK of Tec1 (asterisks indicate phosphorylation at threonine 273 and 276). The figure displays the fragment ions of the peptide (parent ion) showing relative intensity of the fragment ions on the *y* axis and mass to charge ratio (m/z) along the *x* axis. The y-ion series of the peptide is indicated in both blue text and highlighted m/z data points (fragment ions), whereas the b-ion series is in red. The fragment ion colored in gold and labeled Phs correlates with the neutral loss of one phosphoric acid from the parent ion (shorter wider gold bar below *x* axis at 674 m/z). All other major ions not highlighted correspond to either single neutral losses from fragment ions or a second neutral loss from the parent ion.

which specificity is maintained during signaling through the mating pheromone MAPK pathway. Pheromone-dependent activation of the Fus3 MAPK leads to phosphorylation and destruction of Tec1, the target transcription factor for the filamentous growth MAPK signaling pathway. This model explains why pheromone signaling does not activate FRE-dependent gene expression and invasive growth despite the fact that a fraction of Kss1 is phosphorylated during the mating response; activation of Kss1 by pheromone signaling and ensuing activation of Tec1 is counterbalanced by the destruction of Tec1. An interesting implication of this circuit design (Figure 7) is that the relative rates of Tec1 activation by mating and filamentation signals versus the rate of Tec1 destruction by mating signaling should determine the biological outcome of pheromone signaling. This may explain the recent observation that basal signaling through the pheromone response pathway affects *FRE-lacZ* expression in the filamentation-defective laboratory strain S288C (Andersson et al., 2004). It is likely that the fidelity of signaling in this system is determined both qualitatively by the structure of the network and quantitatively by the kinetics of particular reactions.

Our data do not rule out additional roles for the Fus3 MAPK in maintaining signaling specificity. Indeed, the hyperphosphorylation of Kss1 observed in pheromone-treated *fus3*Δ cells is not explained by the effects of Fus3 on Tec1 destruction reported here. Consistent with another role for Fus3 in specificity, we observed that the hyperinvasive phenotype of the phosphoacceptor mutants described here is not strong as that of the *fus3*Δ mutant (Figure 5). Moreover, our recent studies of Ste5, the multikinase scaffold protein of the pheromone response pathway, show that it promotes signaling speci-

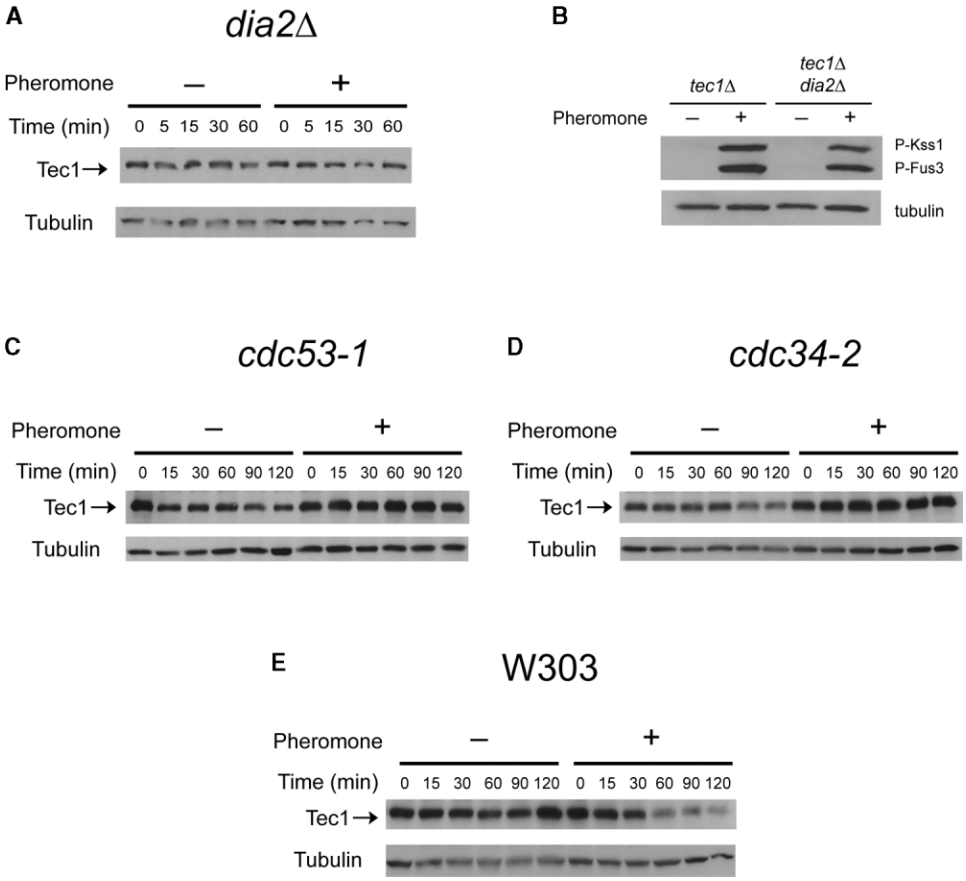

Figure 5. Tec1 Degradation Requires the SCF^{Dia2} Ubiquitin Ligase

(A) *dia2*Δ time course experiment. Immunoblot of cell extracts from *dia2*Δ of the Σ1278b strain background grown at 30°C and either treated or not with α-pheromone for the indicated times. Blots were probed with anti-myc to visualize myc-Tec1 and reprobed with an anti-α-tubulin antibody as a loading control. Strain is isogenic to that shown in (B).

(B) MAPK phosphorylation analysis. Cells of the indicated genotypes were treated or not with mating pheromone for 15 min and analyzed for MAPK phosphorylation by immunoblotting using anti-phospho-ERK2 antibodies as described (Sabbagh et al., 2001). Blots were reprobed with anti-α-tubulin to control for loading.

(C) *cdc53-1*^{ts} time course experiment. Strain (W303 background) was shifted to 37°C for 2.5 hr, but otherwise analyzed as in (A).

(D) *cdc34-2*^{ts} time course experiment. Strain (W303 background) was analyzed as in (C).

(E) Wild-type strain of the W303 strain background. Strain was analyzed as in (E).

ficity at the level of the Ste7 MEK, demonstrating an additional level of specificity in this system (M.A.S. and H.D.M., unpublished observations).

Phosphorylation of the mammalian c-Jun transcription factor by the JNK MAPK has recently been shown to promote its degradation by an SCF ubiquitin ligase (Nateri et al., 2004; Wertz et al., 2004). Thus, the theme of MAPK- and SCF-induced destruction of transcription factors that lie at the terminus of MAPK signaling pathways appears to be conserved in eukaryotic signaling. Phosphorylation and destruction of a transcription factor for a competing pathway offers a general mechanism by which signaling pathways can maintain specificity yet share components. Such crosspathway inhibition could play a critical role in preventing inappropriate signaling characteristics of human malignancies. Intriguingly, Fbw7, an F box protein involved in SCF-dependent c-Jun degradation, is mutated in multiple types of cancers (Moberg et al., 2001; Spruck et al., 2002; Strohmaier et al., 2001).

Experimental Procedures

Yeast Strains, Plasmids, and Epitope Tags

Strains used in this paper are of the Sigma 1278b background unless otherwise noted and are listed in Supplemental Table S1 available at http://www.cell.com/cgi/content/full/119/7/991/DC1/. Plasmids used in this study are listed in Supplemental Table S2 (available on *Cell* website). Epitope tagged-alleles used in this study are as follows. *myc-TEC1* alleles contain six tandem copies of a c-Myc epitope at the N terminus. *FLAG-TEC1* alleles contain three tandem copies of the FLAG epitope at the N terminus. All *TEC1* alleles were expressed from their natural promoter on *CEN* plasmids. *FUS3-FLAG* alleles contain three tandem copies of the FLAG epitope at their C termini. The *DIA2-myc* allele contains 13 tandem copies of the myc epitope at the C terminus.

β-Galactosidase Assay

Cells were grown to log-phase and split into two cultures. One was left untreated while the other is treated with 5 μM α-pheromone. Both were grown at 30°C for 2 additional hours prior to collection for assaying β-galactosidase activity. One mL of cells were harvested from each culture and washed once with Z buffer (60 mM Na_2HPO_4, 40 mM $Na_2H_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, and 38 mM

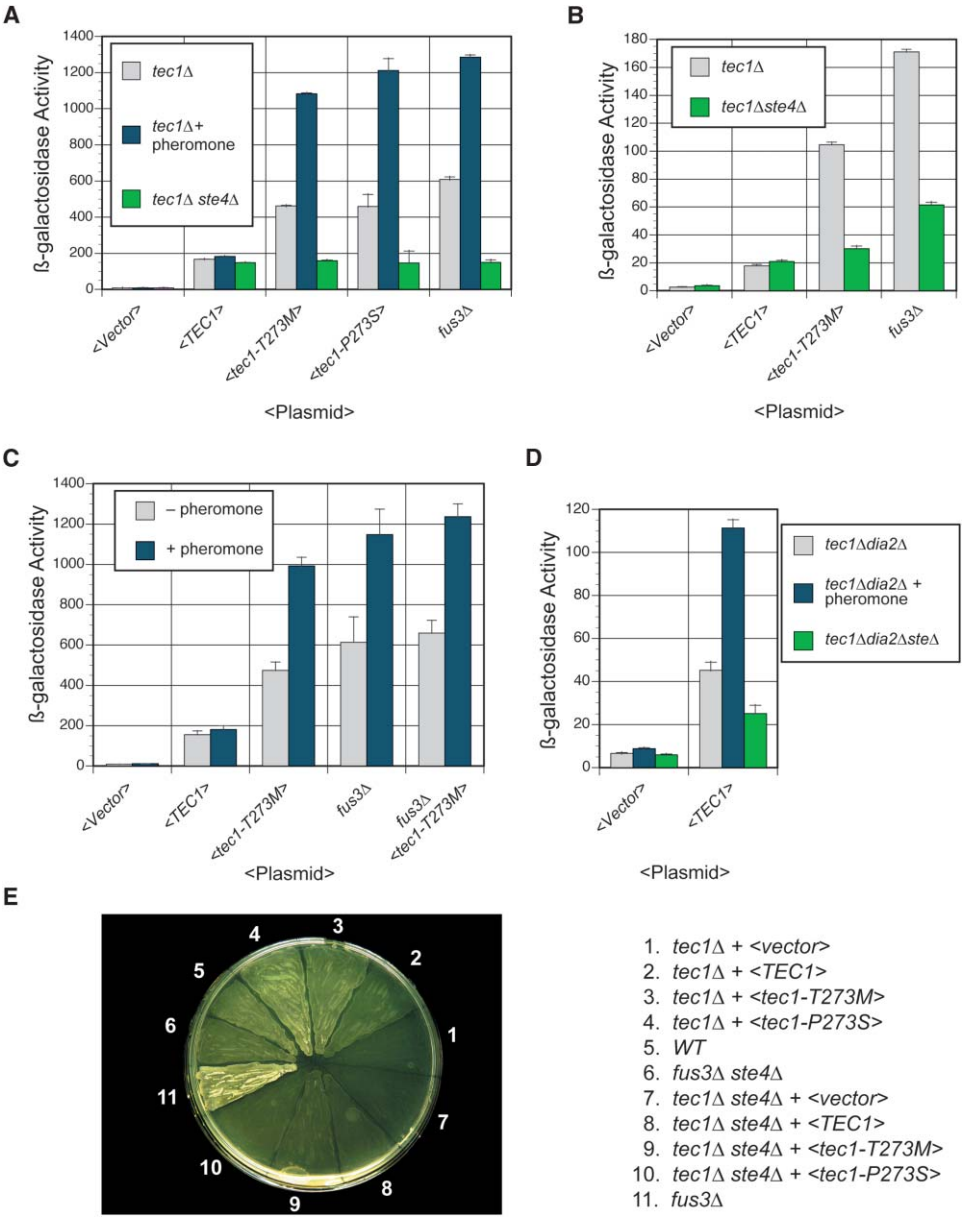

Figure 6. Tec1 Degradation Is Required for Signaling Specificity

(A) *FRE(TEC1)::lacZ* expression. Shown are the β-galactosidase activities of *tec1*Δ and *tec1*Δ*ste4*Δ cells transformed with a centromeric plasmid expressing *TEC1* allele indicated in brackets and *pFRE(TEC1)::lacZ* (first four sets of columns). The β-galactosidase activities of *fus3*Δ and *fus3*Δ*ste4*Δ cells transformed with *pFRE(TEC1)::lacZ* (last set of column) also shown. Exponentially growing cultures were split into two and 5 μM α-pheromone was added to one. Both were then grown at 30°C for two additional hours prior to harvesting of cells.

(B) *pPGU1::lacZ* expression. Shown are the β-galactosidase activities of *tec1*Δ and *tec1*Δ*ste4*Δ cells transformed with a centromeric plasmid expressing a Tec1 allele as indicated in the brackets and a plasmid encoding a full-length *PGU1* promoter::*lac Z* fusion (first three sets of columns). The β-galactosidase activities of *fus3*Δ and *fus3*Δ*ste4*Δ cells transformed with *pPGU1::lacZ* (last set of columns) are also shown. Expression assayed as in (A).

(C) *FRE(TEC1)::lacZ* expression in double mutants. Strains of the indicated genotypes were analyzed as in (A).

(D) *FRE(TEC1)::lacZ* expression in *dia2*Δ mutants. Strains of the indicated genotypes were analyzed as in (A).

(E) Haploid invasive growth assays. Strains of the indicated genotypes were patched, allowed to grow for 2 days at 30°C, washed under a tap, and photographed.

β-mercaptoethanol [BME]). Cells were then resuspended in 500 μL of the same buffer and 100 μL to 300 μL aliquots were taken for assaying β-galactosidase activity. Cell aliquots were permeabilized by two freeze-thaw cycles and then brought to a final volume of 800 μL. 200 μL of 4 mg/mL *o*-nitrophenyl-β-D-galactopyranoside was added to each sample and the reactions were stopped with 400 μL 1 M Na_2CO_3 when solution turned yellow. The optical density at 420 nm was taken of the supernatant following centrifugation to pellet cell debris. β-galactosidase activity was calculated by the following equation: (1000 × $O.D._{420}$)/(time (min) × volume of cells

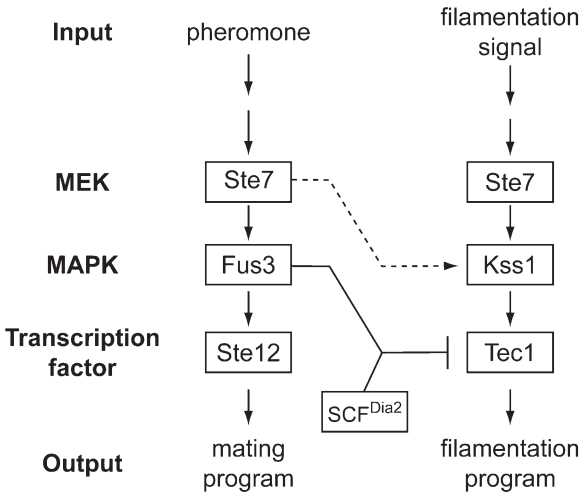

Figure 7. Circuitry of MAPK Signaling

Pheromone-dependent activation of the mating-specific MAPK Fus3 leads to phosphorylation and SCF^{Dia2}-dependent destruction of the filamentation-specific transcription factor Tec1. This cross-pathway inhibition is a mechanism by which the filamentation response can be inhibited during pheromone signaling despite some activation of the filamentation-specific MAPK Kss1.

(mL) × O.D.$_{600}$). The O.D.$_{600}$ refers to the optical density of the cells upon resuspension in 500 μL of buffer. All experiments were done in triplicate with three separate transformants of each strain.

Haploid Invasive Growth Assay

Transformants were patched onto YPAD plates such that a thin layer of cells covered the plate. Plates were incubated at 30°C for two days prior to washing under the tap to distinguish invasive growth. Plates were photographed immediately before and after wash.

Pheromone Time-Course Experiments

Σ*1278b Strains*

*tec1*Δ cells were transformed with a centromeric plasmid expressing wild-type or mutant myc-Tec1 alleles. In the case of the *fus3-K42R* time course, *tec1*Δ*fus3*Δ cells were also transformed with a centromeric plasmid carrying the *fus3-K42R* allele. Cells were grown to early log phase at 30°C and a sample was collected for the zero minute time point. The culture was then split with one half treated with 5 μM α pheromone. Three O.D. units of cells were harvested per time point. Cell were collected by centrifugation and snap-frozen.

Following resuspension in sample buffer with Sigma fungal protease inhibitor cocktail and Sigma phosphatase inhibitor cocktails 1 and 2, cells were lysed by two repetitions of boiling for 2 min and vortexing with 0.5 mm zirconia/silica beads (Biospec Producs) for 2 min. Following centrifugation at 14,000 rpm for 10 min, supernatants were collected. 15 μL of each sample was resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose (0.35 amps for 2.5 hr). Membranes were probed with anti-myc primary antibody (9E10, Covance) and anti-mouse HRP-conjugated secondary (Biorad). Blots were developed with SuperSignal West Pico chemiluminescent substrate (Pierce) and chemoluminescence was visualized using film. To control for loading variation, blots were stripped and reprobed with anti-α-tubulin (Abcam ab6161) and anti-rat HRP-conjugated secondary antibody (Jackson Laboratories).

W303 Strains

Wild-type W303 *cdc34-1,* and *cdc53-2* strains transformed with a centromeric plasmid expressing myc-Tec1 were grown at 22.5° to early log and shifted to the nonpermissive temperature of 37° for 2.5 hr prior to proceeding with the pheromone treatment time course as described above. Lysate preparation and Western methods are same as stated above.

Coimmunoprecipitation Experiments

Yeast extracts were made from 200 mL of cells grown in SC-Ura media. Cultures were split, and half was treated with 5 μM α pheromone for 15 min. Cells were harvested at O.D.$_{600}$ 0.5–0.7, collected by centrifugation, and snap-frozen. Pellets were resuspended in 200 μL of TENNI buffer (50 mM Tris-HCl [pH 7.4], 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% Igepal, Sigma protease inhibitor cocktail, Sigma phosphatase inhibitor cocktails 1 and 2) and zirconia/silica beads were added. Cells were disrupted in a Mini-Beadbeater 8 (Biospec Products) by four cycles of disruption at full power for 1 min, cooling on ice between cycles. Beads were washed in 200 μL TENNI buffer; wash was added to extract. Cell debris was removed by centrifugation at 14,000 rpm at 4°C.

Immunoprecipitations were performed by addition of 1μL of anti-FLAG M2 antibody (Sigma) to 1 mg of extract and incubation on ice for 1 hr. Protein G-Sepharose (Pharmacia) was washed twice in TENNI buffer, and 10 μL was added followed by rocking for 1 hr at 4°C. The slurries were washed 4 times with 500 μL of TENNI buffer and finally boiled in 15 μL sample buffer. The IP and 25 μg of the whole-cell extract were resolved by SDS-PAGE on 10% minigels. Immunoblotting was performed as above.

Recombinant Protein Purification

pMBP-Tec1-FLAG was expressed in *E. coli* BL21 grown in 2X YT containing 100 μg/mL of carbenicillin induced for 4 hr with 0.1 mM IPTG at 24°C at O.D.$_{600}$ 0.6. Cells were lysed by sonication in buffer A (20mM Tris-HCl [pH 7.4], 0.4 M NaCl, 0.1% Triton-X 100, 10 mM EDTA, 10 mM EGTA, 10 mM BME, 1 mM AEBSF, 5 μg/mL leupeptin). Cell debris was pelleted for 20 min at 16,000 rpm at 4°C. 250 μL anti-FLAG-M2 agarose was washed twice in buffer A, added to the clarified extract and the mixture was rocked at 4°C for 4 hr. Beads were washed twice with 10 mL of buffer A and twice with BC300 (20 mM Tris-HCl 7.4, 300 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.1% Igepal). Purified protein was eluted from the agarose by 4 incubations with 500 μL of FLAG peptide (0.2 mg/mL final concentration) in BC300 and eluants were pooled. SDS-PAGE and Coomassie blue staining revealed a single band corresponding to MBP-Tec1-FLAG.

In Vitro Kinase Assays

Fus3-FLAG and Fus3-K42R-FLAG were purified from pheromone-treated cells as described in the coimmunoprecipitation section. After purification, the slurry was additionally washed twice in 500 μL of kinase buffer (50 mM Tris-HCl [pH 7.4], 20 mM $MgCl_2$, 1 mM DTT, Sigma fungal protease inhibitor cocktail, Sigma phosphatase inhibitor cocktails 1 and 2). The slurry was preincubated with 200 ng of purified Tec1 in a total volume of 60 μL for 5 min at 25°C. Kinase reactions were initiated by the addition of 5 μL reaction cocktail (1× kinase buffer, 2 μCi/μL [γ-^{32}P]ATP, 10 μM ATP) and incubated for 15 min at 30°C. The reaction was stopped by addition of 35 μL of 3× sample buffer and boiling. The reactions were resolved by SDS-PAGE and analyzed by autoradiography. Samples for mass spectrometry were generated in similar fashion except that Fus3 was immunoprecipitated from pheromone-treated cells harboring a high copy plasmid expressing Fus3-FLAG, no labeled nucleotides were used, the ATP concentration was raised to 1 mM, 400 ng of Tec1 fusion protein was used, and the incubation time was extended to 1 hr.

Isolation and Characterization of Tec1 Ubiquitin Conjugates

*tec1*Δ cells were transformed with a centromeric plasmid expressing 3X-FLAG-Tec1 and a plasmid carrying an inducible (*pCUP1*) synthetic His6-myc-ubiquitin K42R, G76A allele. Cultures were induced during log phase growth for ubiquitin expression with 250 uM (final concentration) of $CuSO_4$. Cultures were induced with copper sulfate for 1 hr before being incubated with pheromone (5 μM final concentration) for another hour. Cells were lysed by bead beating under denaturing conditions (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 20 mM Tris [pH 8.0], Sigma fungal protease inhibitor cocktail, 10 mM N-ethylmaleimide, 1 mM PMSF). Lysates were incubated with Ni-NTA (Qiagen) agarose beads for 1.5 hr at 4°C. Ni-NTA beads were washed in batch four times in denaturing lysis buffer followed by four times in wash buffer (50 mM Tris [pH 8.0], 500 mM NaCl) before

elution buffer (100 mM Tris [pH 6.8], 1% SDS, 100 mM EDTA, 100 nM DTT) was applied. Half of the elution was resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose (0.35 A for 2.5 hr), and probed with anti-FLAG primary antibody (M2, Sigma) and anti-mouse HRP conjugated secondary antibody (BioRad). The blot was developed with ECL solution (Pierce) and visualized on film.

Mass Spectrometry

The in vitro kinase reactions were precipitated by the addition of TCA to 20%. The precipitate was washed two times with acetone, dried via speedvac (Savant), and resuspended in 8 M urea and 100 mM Tris-HCl [pH 8.5]. The solubilized protein was reduced by the addition of TCEP to 5 mM, followed by the carboxyamidomethylation of cysteines using 10 mM iodoacetamide. The concentration of urea was then diluted 2-fold (to 4 M) by the addition of an equal volume of 100 mM Tris-HCl [pH 8.5]. Sequencing grade modified trypsin (Promega) was added at ~1:50 enzyme to substrate ratio (wt:wt) and incubated at 37°C for 12–16 hr. The digestion was stopped by the addition of formic acid to 4% and subsequently loaded on a 15 cm long microcapillary fused silica column (100 μm i.d.) packed with 5 μm C-18 resin (Aqua, Phenomenex). LC-MS/MS was then carried out as described (McDonald et al., 2002). MS/MS spectra were analyzed with the database search algorithm SEQUEST using a translated ORF database from *S. cerevisiae* with the sequence of MBP-Tec1-FLAG added, where phosphorylation at serine, threonine, and tyrosine were differentially considered. The spectra that were assigned to peptides by SEQUEST were then filtered using previously described criteria (Link et al., 1999).

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