Rts1-protein phosphatase 2A antagonizes Ptr3-mediated activation of the signaling protease Ssy5 by casein kinase I

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ABSTRACT **Ligand-induced conformational changes of plasma membrane receptors initiate signals that enable cells to respond to discrete extracellular cues. In response to extracellular amino acids, the yeast Ssy1-Ptr3-Ssy5 sensor triggers the endoproteolytic processing of transcription factors Stp1 and Stp2 to induce amino acid uptake. Activation of the processing protease Ssy5 depends on the signal-induced phosphorylation of its prodomain by casein kinase I (Yck1/2). Phosphorylation is required for subsequent Skp1/Cullin/Grr1 E3 ubiquitin ligase–dependent polyubiquitylation and proteasomal degradation of the inhibitory prodomain. Here we show that Rts1, a regulatory subunit of the general protein phosphatase 2A, and Ptr3 have opposing roles in controlling Ssy5 prodomain phosphorylation. Rts1 constitutively directs protein phosphatase 2A activity toward the prodomain, effectively setting a signaling threshold required to mute Ssy5 activation in the absence of amino acid induction. Ptr3 functions as an adaptor that transduces conformational signals initiated by the Ssy1 receptor to dynamically induce prodomain phosphorylation by mediating the proximity of the Ssy5 prodomain and Yck1/2. Our results demonstrate how pathway-specific and general signaling components function synergistically to convert an extracellular stimulus into a highly specific, tuned, and switch-like transcriptional response that is critical for cells to adapt to changes in nutrient availability.**

Monitoring Editor
Thomas D. Fox
Cornell University

Received: Jan 9, 2013
Revised: Feb 15, 2013
Accepted: Feb 20, 2013

INTRODUCTION

Cells sense and respond to a multitude of extracellular and intracellular cues. Highly specific signaling pathways ensure that distinct stimuli evoke appropriate responses. Deciphering mechanisms that govern the fidelity of signaling pathways is fundamental to understanding how cells control growth and proliferation. The Ssy1-Ptr3-Ssy5 (SPS) signaling pathway in *Saccharomyces cerevisiae* provides a useful paradigm. In response to extracellular amino acids, this pathway induces the expression of a limited set of genes encoding broad-specificity amino acid permeases that function at the plasma membrane to facilitate amino acid uptake (reviewed in Ljungdahl and Daignan-Fornier, 2012). Accordingly, this pathway is essential for growth of auxotrophic cells lacking the ability to synthesize amino acids. An interesting characteristic of the SPS signaling pathway is that a highly specific output is generated by the orchestrated recruitment of general signaling components, including the plasma membrane–localized casein kinases Yck1 and Yck2, the Skp1/Cullin/F-box (SCF)^{Grr1} E3 ubiquitin ligase complex, and the 26S proteasome, which participate in diverse regulatory events within cells (Gross and Anderson, 1998; Willems *et al.*, 2004; Wolf, 2004; Knippschild *et al.*, 2005; Jonkers and Rep, 2009). The inherent constitutive and promiscuous activity of general factors raises fundamental questions regarding how tight, stimulus-dependent regulation of signal transduction can be achieved.

The SPS pathway triggers the induced expression of amino acid permeases via the endoproteolytic activation of two latent transcription factors, Stp1 and Stp2 (Stp1/2; Andréasson and Ljungdahl,

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-01-0019) on February 27, 2013.

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Abbreviations used: AD, activation domain; β-gal, β-galactosidase; DBD, DNA-binding domain; HA, hemagglutinin; MM, 2-{[({[(4-methoxy-6-methyl)-1,3,5-triazin-2-yl]-amino}carbonyl)amino]-sulfonyl}-benzoic acid; PP2A, protein phosphatase 2A; SCF, Skp1/Cullin/F-box; SD, synthetic minimal dextrose; SPS, Ssy1-Ptr3-Ssy5; vc, vector control; WT, wild type; YPD, yeast extract/peptone/dextrose.

2002). Accordingly, at the heart of this pathway lies the Stp1/2-activating protease Ssy5 (Abdel-Sater *et al.*, 2004; Andréasson *et al.*, 2006; Poulsen *et al.*, 2006; Pfirrmann *et al.*, 2010). Ssy5 exhibits homology to chymotrypsin-like serine proteases and is expressed as a zymogen. On folding, Ssy5 cleaves itself into an N-terminal prodomain and a C-terminal catalytic (Cat) domain. Of importance, the prodomain and Cat domain remain noncovalently associated, and the prodomain functions as an inhibitor that effectively masks the transcription factor processing activity of the Cat domain (Andréasson *et al.*, 2006; Pfirrmann *et al.*, 2010). On amino acid induction, a degron motif within the prodomain is phosphorylated at multiple serine/threonine residues by the pleiotropic Yck1/Yck2 kinases (Yck1/2; Omnus *et al.*, 2011). The hyperphosphorylated degron is recognized by the SCF^{Grr1} ubiquitin E3 ligase complex, leading to prodomain ubiquitylation (Abdel-Sater *et al.*, 2011; Omnus *et al.*, 2011). As a consequence, the polyubiquitylated prodomain is degraded by the 26S proteasome. Thus the phosphorylation-dependent degradation of the inhibitory prodomain induced by extracellular amino acids unfetters the endoproteolytic activity of the Ssy5 Cat domain (Pfirrmann *et al.*, 2010; Omnus *et al.*, 2011).

Protein phosphorylation is often reversible; hence a change of protein function can be controlled by the opposing activities of protein kinases and phosphatases. Thus, in principle, signaling events can modulate the activity of either a kinase or a phosphatase to generate a signaling output. Examples of highly specific phosphorylation-dependent signaling pathways include the MAP kinase pathway regulating yeast mating (reviewed in Malleshaiah *et al.*, 2010) and the cyclin-dependent kinases governing cell cycle progression (reviewed in Domingo-Sananes *et al.*, 2011). With respect to the SPS sensing pathway, proper control of Ssy5 activity depends on Rts1 (Eckert-Boulet *et al.*, 2006; Liu *et al.*, 2008), a regulatory subunit of protein phosphatase 2A (PP2A; Zhao *et al.*, 1997). Deletion of *RTS1* leads to constitutive Stp1/2 processing, resulting in SPS sensor–regulated promoter activation even in the absence of amino acid induction (Eckert-Boulet *et al.*, 2006; Liu *et al.*, 2008). Hence, although the underlying mechanism has not been elucidated, data suggest that PP2A exerts a negative regulatory function.

The Yck1/2 kinases and PP2A can modify many different substrate proteins. This property enables their involvement as general factors in multiple signaling pathways (Robinson *et al.*, 1992; Gross and Anderson, 1998; Marchal *et al.*, 2002; Moriya and Johnston, 2004; Knippschild *et al.*, 2005; Harvey *et al.*, 2011; Pracheil *et al.*, 2012). Apparently, their participation in the SPS signaling pathway is coupled to discrete events that modulate the targeting of their enzymatic activity to pathway specific components. Accordingly, core pathway components may function as adapters that promote interactions between general and pathway-specific factors (Rossio *et al.*, 2010). In addition to merely providing a passive scaffolding function, adaptors may actively participate in signal propagation by promoting or stabilizing signaling conformations of effector proteins (Good *et al.*, 2009). In such instances, the dynamic participation of adaptor proteins in controlling the spatial proximity between general and specific signaling components directly contributes to the maintenance of pathway integrity and the fidelity of signal transduction.

The signaling events leading to Ssy5 activation exhibit an absolute requirement for Ptr3, the least understood component of the SPS sensor (Barnes *et al.*, 1998; Klasson *et al.*, 1999; Forsberg and Ljungdahl, 2001; Poulsen *et al.*, 2005; Liu *et al.*, 2008). Available information suggests that Ptr3 can interact with Ssy1, Ssy5, and itself (Bernard and André, 2001; Liu *et al.*, 2008). Consistently, the C-terminal half of Ptr3 contains sequences homologous to WD40 repeats that are known to fold into a propeller-like structure capable of mediating protein–protein interactions (Neer *et al.*, 1994; Pashkova *et al.*, 2010; Ritterhoff *et al.*, 2010; Cartier *et al.*, 2011). Ptr3 has been reported to be inducibly phosphorylated in a Yck1/2-dependent manner (Liu *et al.*, 2008); however, the sites of phosphorylation are not known. The significance of Ptr3 phosphorylation and a mechanistic understanding of the requirement of Ptr3 in promoting Ssy5-dependent Stp1/2 processing remains elusive.

Here, on the basis of our insights regarding the critical role of Ssy5 prodomain phosphorylation in SPS-sensor signaling (Omnus *et al.*, 2011), we investigated the signaling events coupling Ssy1 receptor function to Ssy5 activation. Specifically, the data provide novel mechanistic details demonstrating that Ptr3 plays a key role as a dedicated adaptor that links the conformational changes of the Ssy1 receptor to the juxtaposition of Yck1/2 and Ssy5, thereby facilitating prodomain phosphorylation. We also show that Rts1-mediated PP2A phosphatase constitutively antagonizes Yck1/2-dependent prodomain phosphorylation, which sets a signaling threshold that effectively mutes Ssy5 activation in the absence of amino acid induction.

RESULTS

Constitutive Rts1-targeted PP2A sets a threshold for phosphorylation-induced Ssy5 activation

Deletion of *RTS1* results in constitutive endoproteolytic processing of Stp1/2 in a manner that retains a strict requirement for a functional SPS sensor (Eckert-Boulet *et al.*, 2006). According to the current understanding of SPS-sensor signaling, the Stp1/2 processing protease Ssy5 is activated by phosphorylation-induced degradation of its inhibitory prodomain (Omnus *et al.*, 2011). We therefore examined whether Rts1 directly controls the phosphorylation status of the Ssy5 prodomain and, as a consequence, its stability. In comparison to wild-type (WT) cells, prodomain levels in *rts1*Δ cells grown under noninducing (–leu) condition and 30 min after induction with leucine (+leu) were significantly lower (Figure 1A, compare lane 1 with lane 3, and lane 2 with lane 4, respectively). These results indicate that Rts1 participates in governing prodomain stability, likely by maintaining the hypophosphorylated state of the Ssy5 phosphodegron in the absence of bona fide amino acid–induced signaling.

To test this possibility, we monitored the status of prodomain phosphorylation under conditions in which phosphorylated forms of prodomain accumulate, that is, in cells lacking Grr1 (Abdel-Sater *et al.*, 2011; Omnus *et al.*, 2011). Grr1 is a component of the SCF^{Grr1} complex that ubiquitylates the phosphorylated prodomain, targeting it for proteasomal degradation (Omnus *et al.*, 2011). In the absence of Grr1, prodomain degradation is blocked, enabling the accumulated phosphorylated species to be visualized. In *rts1*Δ *grr1*Δ double-mutant cells, and in contrast to *grr1*Δ cells, slower-migrating phosphorylated prodomain species were readily observed under noninducing conditions (Figure 1B, compare lane 3 with 1). On leucine induction, phosphorylated and extensively hyperphosphorylated prodomain species accumulated in both *grr1*Δ and *rts1*Δ *grr1*Δ cells (Figure 1B, lanes 2 and 4, respectively). Of note, in comparison to *grr1*Δ cells, the levels of hyperphosphorylated prodomain that accumulate were significantly higher in *rts1*Δ *grr1*Δ cells, indicating that Rts1 mediates an antagonistic dampening effect even under signaling conditions. These results demonstrate that Rts1 contributes to maintaining the hypophosphorylated state of the prodomain in the absence of signaling and that amino acid–induced signaling increases kinase access to the prodomain phosphodegron in a manner that overrides the constitutive Rts1-dependent phosphatase.

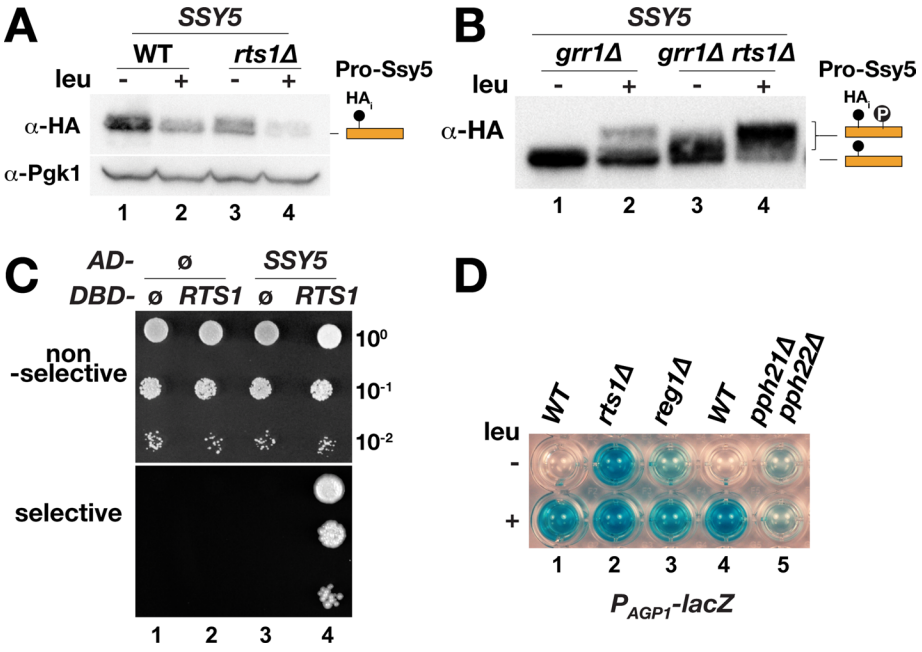

FIGURE 1: Ssy5 prodomain phosphorylation is modulated by Rts1-PP2A. (A) Immunoblot analysis of extracts from BY4741 (WT) and BY4741 (*rts1*Δ) carrying plasmids pCJ353 (*SSY5-42-HA$_6$*) and pAB1 (to enable prototrophic growth). (B) Immunoblot analysis of extracts from BY4741 (*grr1*Δ) and BY4741 (*grr1*Δ *rts1*Δ) carrying plasmids as in A. Immunoreactive forms of phosphorylated and nonphosphorylated Ssy5 prodomain species are indicated at their corresponding positions of migration. (C) Directed two-hybrid analysis of Rts1 and Ssy5 interactions. Plasmid pDO185 (*DBD-RTS1*) or pGBKT7 (*DBD*; ∅), together with pACTII (*AD*; ∅) or pACTII-Ssy5 (*AD-SSY5*), was introduced into AH109. Growth of transformants was assessed on nonselective and selective media. (D) BY4741 (WT), BY4741 (*rts1*Δ), and BY4741 (*reg1*Δ) carrying pAGP1-lacZ and pAB1, as well as BY4742 (WT) and DC152 (*pph21*Δ*pph22*Δ) carrying pAGP1-lacZ, pAB1, and pRS317, were grown in SD medium with or without leucine (leu). The levels of β-gal in permeabilized cells were assessed by X-Gal staining.

Rts1 acts as a specificity factor that guides catalytic PP2A subunits Pph21 and Pph22 to their substrates (Zhao *et al.*, 1997). We examined whether Rts1 can physically associate with Ssy5 by employing a directed two-hybrid assay. Only when introduced together did the bait DNA-binding domain (*DBD*)-*RTS1* and the prey activation domain (*AD*)-*SSY5* constructs facilitate robust growth of the host strain on media selective for expression of the two-hybrid interaction reporters, that is, P$_{GAL1}$-*ADE2* and P$_{GAL1}$-*HIS3* (Figure 1C). These observations suggest that Rts1 and Ssy5 interact, a finding consistent with the known role of Rts1 in defining the substrate specificity of PP2A.

Next we examined whether inactivation of the catalytic PP2A components would result in constitutive Stp1 processing; we monitored *β*-galactosidase (*β*-gal) expression from the Stp1-regulated *AGP1* promoter (P$_{AGP1}$-*lacZ*; Figure 1D; Iraqui *et al.*, 1999). Cells lacking *RTS1* (*rts1*Δ, row 2) or both *PPH21* and *PPH22* (*pph21*Δ *pph22*Δ, row 5) exhibited constitutive promoter activity independent of leucine induction. Wild-type cells exhibited proper P$_{AGP1}$-regulated expression, that is, no *β*-gal expression in the absence of signaling (–leu) and robust *β*-gal expression upon leucine induction (+leu; Figure 1D, rows 1 and 4). The relatively low level of constitutive *β*-gal expression in *pph21*Δ*pph22*Δ cells is likely due to reduced growth (Sneddon *et al.*, 1990). Reg1, generally acknowledged to be the major regulatory subunit of protein phosphatase 1, was recently shown to enhance interactions between Rts1 and catalytic PP2A subunits (Castermans *et al.*, 2012). Consistent with Reg1 enhancing PP2A activity, *reg1*Δ-mutant cells exhibited constitutive *β*-gal expression (Figure 1D, row 3). These results indicate that Rts1 associates with Ssy5 and together with Reg1 functions to direct the PP2A to the Ssy5 prodomain.

Ptr3 is required for signal-dependent phosphorylation of Ssy5

Our finding that the Ssy5 prodomain is a constitutive substrate of Rts1-dependent PP2A raised questions as to how amino acid–induced signaling enables the Yck1/2 kinase to surmount the antagonistic activity of the phosphatase. We focused on the role of Ptr3. Ptr3 has two domains (Figure 2A): an N-terminal domain (amino acids [aa] 1–300) with small and dispersed regions of sequence similarity to fungal homologues and a well-conserved C-terminal domain (aa 301–678) with WD40-like repeats. To place Ptr3 function within the cascade of SPS-sensor signaling reactions, we asked whether Ptr3 is required for induced Ssy5 prodomain phosphorylation. We monitored prodomain phosphorylation in *grr1*Δ cells; the slower-migrating phosphorylated form of the prodomain was only observed in leucine-induced cells harboring Ptr3 (*PTR3*) and not in cells lacking Ptr3 (vector control [vc]; Figure 2B, compare lanes 1–4). Next we tested whether the previously described loss-of-function mutant of Ptr3 (*ptr3-T525A*), which harbors a threonine-to-alanine substitution at position 525 in the C-terminal WD40-like domain (Figure 2A; Liu *et al.*, 2008), affected prodomain phosphorylation. As in cells lacking Ptr3, we could not detect phosphorylated prodomain species in cells expressing *ptr3-T525A* (Figure 2B, lane 6). These results indicate that Ptr3 facilitates prodomain phosphorylation in response to leucine induction and that the T525A mutation interferes with this function.

The ability to homo-oligomerize is essential for Ptr3 function

Next we examined whether the loss of function resulting from the T525A mutation could be linked to the inability of mutant protein to associate with known Ptr3 interaction partners, that is, the N-terminal cytoplasmic domain of Ssy1, Ssy5, and itself (Bernard and André, 2001; Liu *et al.*, 2008). We exploited a directed two-hybrid approach and analyzed interactions using the Ssy1 N-terminus (Ssy1$_{NT}$) and Ptr3 as bait (DBD; Figure 2C, left and right, respectively) and Ssy5, Ptr3 and the mutant ptr3-T525A as prey constructs (AD). As previously reported (Liu *et al.*, 2008), the N-terminal domain of Ssy1 interacted with both Ssy5 and Ptr3; robust growth was observed on media selective for expression of the two-hybrid interaction reporters (Figure 2C, dilutions 4 and 6). Of importance, Ssy1$_{NT}$ interacted with the ptr3-T525A mutant protein as well as the wild-type Ptr3 (Figure 2C, dilution 8). Also consistent with previous findings (Bernard and André, 2001), the Ptr3 bait construct interacted with Ssy5 and wild-type Ptr3 (Figure 2C, dilutions 12 and 14). Strikingly, the Ptr3 bait construct did not interact with the mutant ptr3-T525A (Figure 2C, dilution 16), indicating that the T525A mutation abolishes the ability of Ptr3 to interact with itself. This and the fact that

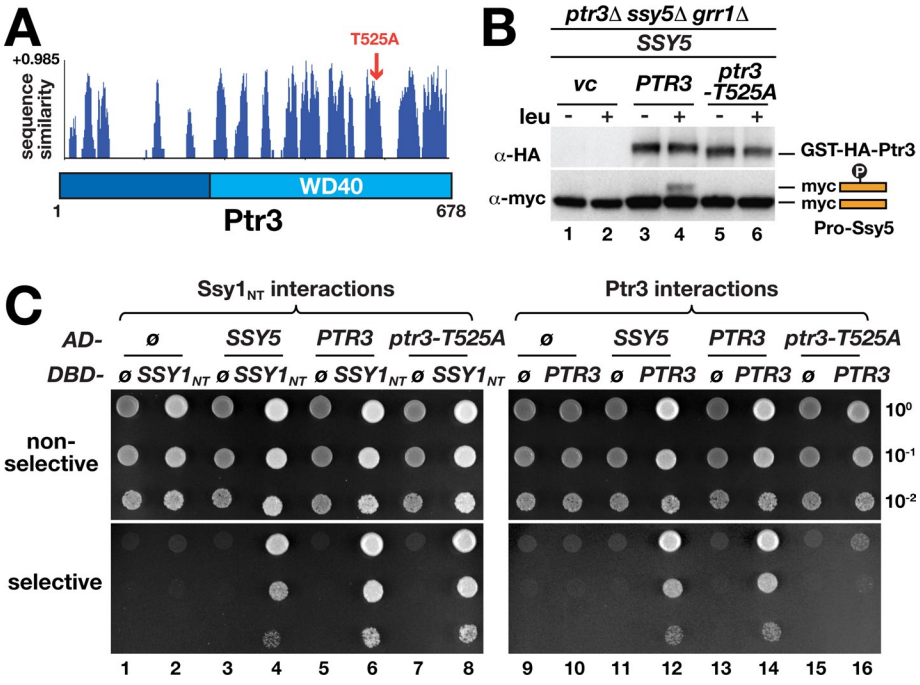

FIGURE 2: Ssy5 prodomain phosphorylation is Ptr3 dependent. (A) Similarity plot (Clustal X) of the aligned protein sequences of fungal Ptr3 orthologues; schematic presentation of *S. cerevisiae* Ptr3 (residues 1–678) depicting the relatively poorly conserved N-terminal (dark blue) and highly conserved C-terminal WD40-like domain (light blue). The position of the T525A loss-of-function mutation is indicated (red arrow). (B) Immunoblot analysis of extracts from CAY307 (*ptr3Δ ssy5Δ grr1Δ*) carrying plasmid pHK048 (*SSY5*) and plasmid pRS317 (*vc*), pDO148 (*PTR3*), or pDO149 (*ptr3-T525A*). Immunoreactive forms of Ptr3, as well as of phosphorylated and nonphosphorylated Ssy5 prodomain species, are indicated. (C) Directed two-hybrid analysis of Ssy5, Ptr3 and ptr3-T525A interactions with the N-terminal domain of Ssy1 (left) and Ptr3 (right). Tenfold dilutions (1–8) were prepared from AH109 carrying plasmid pGBKT7-SSY1N (*DBD-SSY1$_{NT}$*) or pGBKT7 (*DBD*; ∅) and either pACTII (*AD*; ∅), pACTII-Ssy5 (*AD-SSY5*), pACTII-Ptr3 (*AD-PTR3*). or pDO189 (*AD-ptr3-T525A*). Similarly, dilutions (9–15) were prepared from AH109 carrying pDO186 (*DBD-PTR3*) or pGBKT7 (*DBD*; ∅) and either pACTII (*AD*; ∅), pACTII-Ssy5 (*AD-SSY5*), pACTII-Ptr3 (*AD-PTR3*), or pDO189 (*AD-ptr3-T525A*). Dilutions of cell suspensions were spotted on nonselective and selective media as in Figure 1C.

the T525A mutation abolishes Ssy5 prodomain phosphorylation suggested that the ability of Ptr3 to homo-oligomerize is essential for signal transduction.

Ptr3 and Ssy5 form a constitutive complex

To assess the role of Ptr3 in facilitating signal-induced prodomain phosphorylation, we first used a split ubiquitin assay to evaluate interactions between Ptr3 and Ssy5. This assay is specifically designed to detect in vivo interactions between proteins without altering their intracellular location, and importantly, allows the dynamic nature of signal-induced interactions to be assessed (see *Materials and Methods*). We fused the N-terminal fragment of ubiquitin (N_{Ub}) harboring the I13A mutation to Ptr3 (*N_{Ub}-PTR3*) and to the oligomerization deficient ptr3-T525A (*N_{Ub}-ptr3-T525A*) and individually coexpressed these constructs with full-length *SSY5*, *SSY5* prodomain (ssy5$_{Pro}$), or catalytic domain (ssy5$_{Cat}$) fused to the C-terminal fragment of ubiquitin linked to a glutathione *S*-transferase (GST)–hemagglutinin (HA) reporter module (*C_{Ub}-GST-HA*; Figure 3A).The cleaved reporter (GST-HA), indicative of a physical interaction, was present in extracts from *ptr3Δ ssy5Δ* cells expressing both Ssy5-C_{Ub}-GST-HA and N_{Ub}-Ptr3 or N_{Ub}-ptr3-T525A independent of whether the cells were grown in the absence or presence of inducing leucine (Figure 3A, lanes 1, 2, 9, and 10) and in cells also lacking Ssy1 (lane 4). The assay faithfully monitors the interaction of Ptr3 with Ssy5 since cleavage of the reporter strictly depended on the presence of Ptr3; the cleaved reporter was not detected when N_{Ub} (vc) was coexpressed with *SSY5-C_{Ub}-GST-HA* (Figure 3A, lane 3). The absence of the cleaved reporter in cells expressing either the prodomain (ssy5$_{Pro}$) or catalytic domain (ssy5$_{Cat}$; lanes 5–8) suggests that only the correctly folded Ssy5 holoenzyme is capable of interacting with Ptr3. The confirmation that the mutant protein encoded by the *ptr3-T525A* allele, which cannot homo-oligomerize, retains the ability to interact with Ssy5 indicates that oligomerization of Ptr3 is not necessary for the interaction with Ssy5. Taken together, these results suggest that Ptr3 and Ssy5 constitutively interact and do so even in the absence of the upstream component Ssy1.

The constitutive nature of the Ptr3 and Ssy5 interaction suggested that signal-transducing events within the SPS sensor do not regulate this interaction. To test this, we fused Ptr3 to the C-terminus of Ssy5 and thereby forced their spatial proximity (Figure 3B). The chimeric construct was introduced into double-mutant *ptr3Δ ssy5Δ* and triple-mutant *ssy1Δ ptr3Δ ssy5Δ* strains, and Stp1 processing was monitored by immunoblotting (Figure 3B, left) and growth on yeast extract/peptone/dextrose (YPD) in the presence of 2-{[({[(4-methoxy-6-methyl)-1,3,5-triazin-2-yl]-amino}carbonyl)amino]-sulfonyl}-benzoic acid (MM; right). MM is an inhibitor of branched-chain amino acid synthesis, and, consequently, the ability to grow in YPD media containing MM requires Stp1/2-induced expression of high-affinity permeases for leucine, isoleucine, and valine (Jørgensen *et al.*, 1998). Growth on YPD plus MM provides a highly sensitive readout of Ssy5 activity; low amounts of Stp1/2 processing result in readily detectable growth (Pfirrmann *et al.*, 2010). The Ssy5–Ptr3 chimera is fully functional and subject to proper regulation in the context of SPS-sensor signaling; Ssy5–Ptr3 supported amino acid–induced Stp1 processing (Figure 3B, left, compare lanes 1 and 2) and robust growth on YPD plus MM (Figure 3B, dilution 1). The activity of the Ssy5-Ptr3 chimera was dependent on Ssy1; in *ssy1Δ ptr3Δ ssy5Δ* cells, leucine induction did not result in Stp1 processing (Figure 3B, left, lane 4) or growth on YPD plus MM (Figure 3B, right, dilution 2). Taken together, these results show that the mere placement of Ptr3 in the vicinity of Ssy5 is not sufficient for its activation. The fact that fusion of Ptr3 to Ssy5 is compatible with regulated signaling is consistent with the possibility that Ptr3 and Ssy5 interact constitutively.

The Ptr3–Ssy5 subcomplex undergoes a conformational change that triggers Ssy5 activation

The finding that Ptr3 and Ssy5 constitutively associate raised the possibility that Ptr3 facilitates Ssy5 phosphorylation by mediating amino acid–induced interactions with Yck1/2. Consistent with this possibility, Ptr3 is itself phosphorylated by Yck1/2 upon amino acid induction in a strictly Ssy1-dependent manner, and Ptr3 phosphorylation

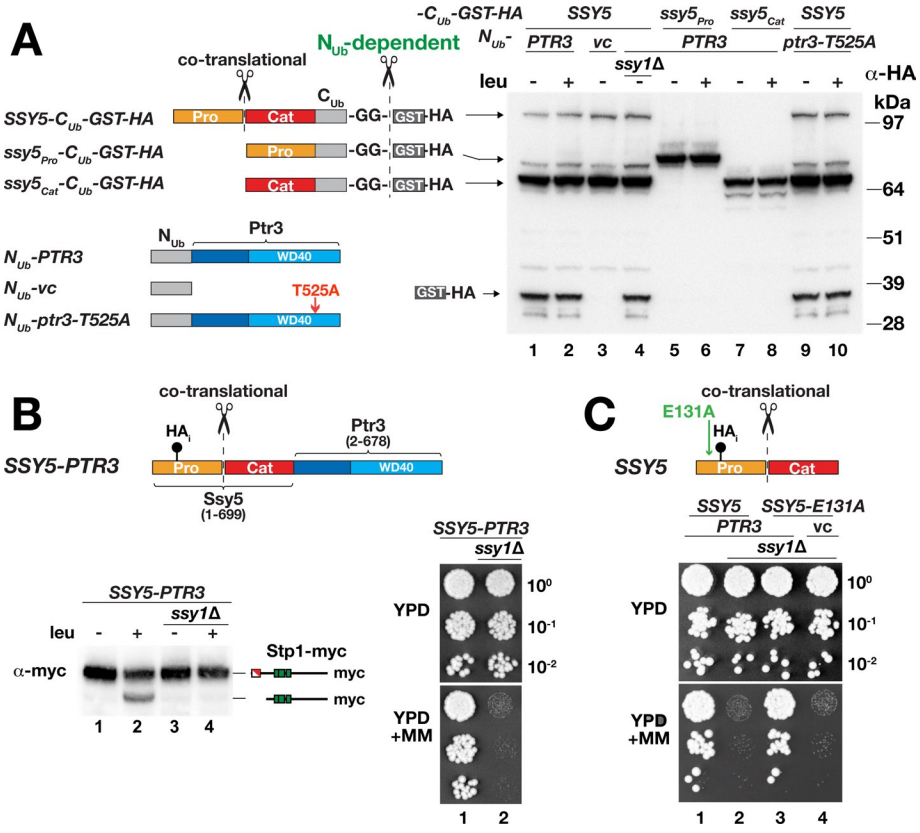

FIGURE 3: Ptr3 and Ssy5 interact constitutively. (A) Schematic diagram of Ssy5 (C_{Ub}) and Ptr3 (N_{Ub}) fusion proteins. Scissors indicate the sites of autolytic processing of Ssy5 (cotranslational) and diagnostic cleavage indicative of split-ubiquitin interactions (N_{Ub} dependent). Immunoblot analysis of extracts from HKY85 (*ptr3Δ ssy5Δ*; lanes 1–3, 5–10) and CAY285 (*ssy1Δ ptr3Δ ssy5Δ*; lane 4) carrying plasmid pDO85 (*SSY5-C_{Ub}-GST-HA*), pDO182 (*$ssy5_{Pro}$-C_{Ub}-GST-HA*), or pDO181 (*$ssy5_{Cat}$-C_{Ub}-GST-HA*), together with plasmid pDO88 (*N_{Ub}*), pDO61 (*N_{Ub}-PTR3*), or pDO94 (*N_{Ub}-ptr3-T525A*). (B) Schematic representation of the Ssy5–Ptr3 fusion protein; the cotranslational autolytic processing site within Ssy5 (scissors), as well as the internal HA tag (HA_i) in the prodomain, are indicated. Immunoblot analysis (bottom left) of extracts from HKY85 (*ptr3Δ ssy5Δ*; lanes 1 and 2) and CAY285 (*ssy1Δ ptr3Δ ssy5Δ*; lanes 3 and 4) carrying plasmids pDO79 (*SSY5-PTR3*) and pCA204 (*STP1-MYC*). Immunoreactive forms of fusion proteins and of Stp1 (full length and processed) are indicated. Growth of strains (bottom right) was assessed on YPD and YPD plus MM media. (C) Schematic representation of the constitutive Ssy5 mutant harboring the E131A mutation in the prodomain (green arrow). Growth of HKY85 (*ptr3Δ ssy5Δ*; dilution 1) and CAY285 (*ssy1Δ ptr3Δ ssy5Δ*; dilutions 2–4) carrying plasmid pSH120 (*SSY5*) or pTP115 (*SSY5-E131A*), and pDO148 (*PTR3*) or pRS317 (*vc*), on YPD and YPD plus MM media.

occurs even in the absence of Ssy5 (Liu *et al.*, 2008). In addition, a selection for mutations in *SSY5* that bypass the requirement of Ssy1 identified the *SSY5-E131A* allele (Pfirrmann *et al.*, 2010), which, in contrast to wild-type *SSY5*, triggers constitutive Stp1 processing and supports growth of a *ssy1Δ* strain on YPD plus MM (Figure 3C, compare dilutions 2 and 3). Significantly, although independent of Ssy1, the constitutive activity of the mutant Ssy5-E131A protein retains the requirement for Yck1/2 and SCF^{Grr1} (Omnus *et al.*, 2011). Thus Ssy5-E131A follows the phosphorylation- and ubiquitylation-dependent activation mechanisms of wild-type Ssy5. Because Ssy5-E131A does not require Ssy1 for its phosphorylation-dependent activation, it is unlikely that Ssy1 directly mediates the physical interaction of Yck1/2 and Ssy5.

To test whether Ptr3 mediates interactions between Yck1/2 and Ssy5, we examined whether Ssy5-E131A requires Ptr3 for its activation. The results indicate that the Ssy1-independent activity of Ssy5-E131A exhibits a strict requirement for the presence of Ptr3; *ssy1Δ ptr3Δ ssy5Δ* cells harboring *SSY5-E131A* only grew on YPD plus MM when Ptr3 was coexpressed (Figure 3C, dilution 4). Taken together, these findings support a model in which amino acid binding to Ssy1 stabilizes a signaling conformation of Ptr3 that provides a binding surface for Yck1/2. Thus Ptr3 has a critical role in recruiting the kinase into spatial proximity of Ssy5. In addition, Ptr3 and Ssy5 conformations and conformational changes induced upon signaling appear to be tightly coupled, consistent with the finding that these proteins form a constitutive subcomplex within the SPS sensor.

Spatial proximity of Yck1 to Ptr3 constitutively activates Ssy5

To address whether amino acid–induced signaling triggers a physical interaction between Yck1/2 and Ptr3, we asked whether the forced spatial proximity of one of these functionally redundant kinases (Robinson *et al.*, 1992; Wang *et al.*, 1992) to Ptr3 would suffice to activate Ssy5. We constructed *YCK1-PTR3*, which encodes a fusion protein with the soluble catalytic domain of Yck1 fused to the N-terminus of Ptr3 (Figure 4A). This fusion construct was introduced into *ptr3Δ* and *ssy1Δ ptr3Δ* strains and Stp1 processing activity was assayed. Strikingly, the expression of *YCK1-PTR3* led to constitutive, Ssy1-independent processing (Figure 4, A, center, lanes 1, 5, and 6, and C, lanes 3, 5, and 6) and supported growth on YPD plus MM (Figure 4B, right, dilutions 1, 3, 5, and 6). Of importance, the constitutive activity of Ssy5 in cells harboring Yck1-Ptr3 was fully dependent on the kinase activity of the chimeric protein; the introduction of the *yck1-PTR3* allele carrying the kinase-inactivating K98R mutation (Wang *et al.*, 1992) prevented constitutive Stp1 processing (Figure 4A, center, lanes 3, 7, and 8) and did not support growth on YPD plus MM (Figure 4B, right, dilution 4). Of note, the *yck1-PTR3* allele expresses a functional Ptr3; it fully complemented the amino acid–induced signaling defects when introduced into a *ptr3Δ* strain (Figure 4A, center, lane 4; right, dilution 2).

We observed that the amount of processed Stp1 triggered by expression of *YCK1-PTR3* in cells harboring a functional SPS sensor increased upon induction of signaling (Figure 4, A, center, compare lane 1 with lane 2, and C, compare lane 3 with lane 4). The enhanced Stp1 processing indicates that even in the context of the fusion to an active kinase, Ptr3 retains its ability to transmit amino acid–induced signals toward Ssy5, likely due to its ability to interact with endogenous Yck1/2. Consistently, the constitutive activity of Ssy5 resulting from expression of *YCK1-PTR3* was the consequence of phosphorylation-induced and ubiquitylation-dependent degradation of its prodomain. In the absence of Grr1, the Yck1–Ptr3 chimera did not support growth on YPD plus MM (Figure 4B, dilution 7). Taken together, our results demonstrate that the placement of

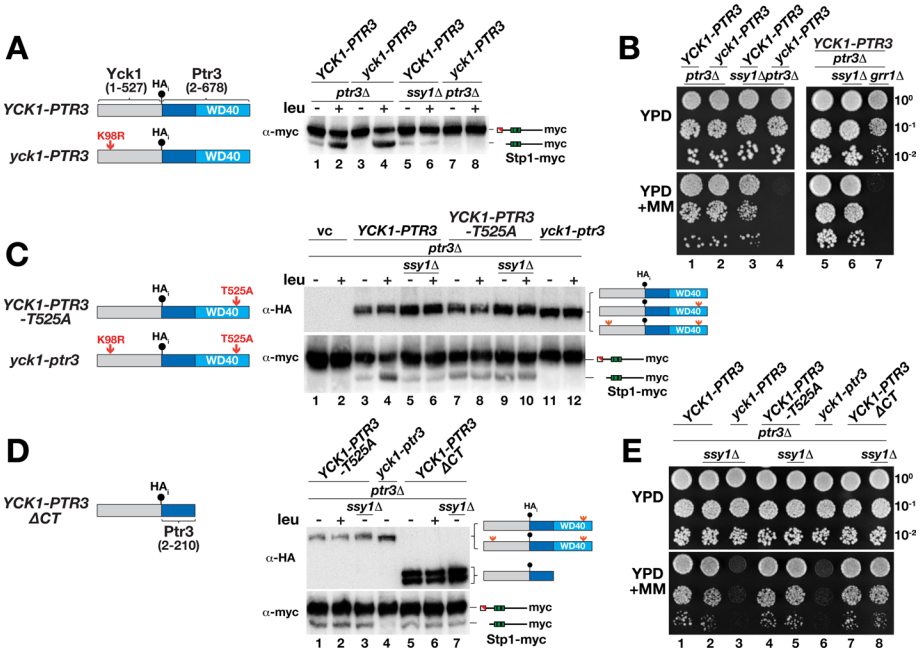

FIGURE 4: A Yck1–Ptr3 fusion protein constitutively induces the Stp1-processing activity of Ssy5. Schematic representation of the Yck1–Ptr3 fusion proteins carrying an internal HA tag (HA_i). Red arrows indicate the positions of the kinase-inactivating K98R mutation (*yck1*) and the T525A mutation within the WD40-like domain of Ptr3. (A) Immunoblot analysis of extracts from HKY31 (*ptr3*Δ) and HKY33 (*ptr3*Δ *ssy1*Δ) carrying plasmids pCA204 (*STP1-MYC*) and pDO84 (*YCK1-PTR3*) or pDO86 (*yck1-PTR3*). (B) Growth of strains as in A (left) and of HKY31 (*ptr3*Δ), HKY33 (*ptr3*Δ *ssy1*Δ), and CAY272 (*ptr3*Δ *grr1*Δ) carrying plasmids pCA204 (*STP1-MYC*) and pDO84 (*YCK1-PTR3*) (right) on YPD and YPD plus MM media. (C) Immunoblot analysis of extracts from HKY31 (*ptr3*Δ; lanes 1–4, 7 and 8, and 11 and 12) and HKY33 (*ptr3*Δ *ssy1*Δ; lanes 5 and 6, and 9 and 10) carrying plasmids pCA204 (*STP1-MYC*) and pRS316 (vc), pDO84 (*YCK1-PTR3*), pDO95 (*YCK1-PTR3-T525A*), or pDO98 (*yck1-ptr3*). (D) Immunoblot analysis of extracts from HKY31 (*ptr3*Δ; lanes 1 and 2 and lanes 5 and 6) and HKY33 (*ptr3*Δ *ssy1*Δ; lanes 3 and 4 and lane 7) carrying plasmids pCA204 (*STP1-MYC*) and pDO95 (*YCK1-PTR3-T525A*), pDO98 (*yck1-ptr3*), or pDO162 (*YCK1-PTR3*Δ*CT*). Immunoreactive forms of the fusion proteins and of Stp1(full length and processed) are indicated. (E) Growth of strains as in D and of strain HKY31 (*ptr3*Δ) carrying plasmids pCA204 (*STP1-MYC*) and pDO86 (*yck1-PTR3*) on YPD and YPD plus MM media.

Yck1 in proximity to Ptr3 suffices to trigger Ssy5 activation, consistent with SPS-sensor signaling regulating the spatial proximity of Yck1/2 to the Ptr3–Ssy5 subcomplex.

The N-terminal domain of Ptr3 mediates interactions with Ssy5

The observation that fusion of Yck1 to Ptr3 constitutively activates Ssy5 independent of Ssy1 allowed us to identify and assign specific functions to elements in Ptr3 required for Ssy5 activation. The T525A loss-of-function mutation in the WD40-like domain of Ptr3 abrogates amino acid–induced Ssy5 phosphorylation; however, the mutant protein retains the ability to interact with Ssy5 (Figure 2, B and C; Liu *et al.*, 2008). The latter finding raised the possibility that elements other than the WD40-like domain mediate Ptr3 and Ssy5 interactions, which we proceeded to test. First, we introduced the T525A mutation into the *YCK1-PTR3* allele and examined whether the modified fusion protein would retain the ability to direct Yck1 to Ssy5 and promote constitutive signaling. Expression of *YCK1-PTR3-T525A* resulted in constitutive Stp1 processing independent of amino acid induction and Ssy1 (Figure 4C, right, lanes 7–10). Stp1 processing depended on an active kinase; in the context of chimera with an inactive kinase (*yck1-ptr3*), Stp1 was not processed (Figure 4C, right, lanes 9 and 10). Next we examined whether the entire WD40-like domain is dispensable for mediating Ptr3 and Ssy5 interactions; we constructed a Yck1 chimera containing only the first 210 amino acids of Ptr3 (*YCK1-PTR3*Δ*CT*). Strikingly, expression of this chimera with the heavily truncated Ptr3 protein resulted in constitutive Stp1 processing independent of Ssy1 at levels indistinguishable from that of the *YCK1-PTR3-T525A* chimera (Figure 4D, center, lanes 5 and 6 and lanes 1 and 2, respectively) and robust growth on YPD plus MM (Figure 4D, right, dilutions 7 and 8 and dilutions 4 and 5, respectively). These data are consistent with the possibility that the first 210 amino acids of Ptr3 mediate interactions with Ssy5.

Ptr3 possesses a conserved LFA motif required for function

To identify sequence elements within the N-terminal domain of Ptr3 that mediate interactions with Ssy5, we compared the amino acid sequences of a set of fungal Ptr3 orthologues (Figure 5A, top). Our analysis identified four regions in the first 210 amino acids of Ptr3 containing stretches of conserved amino acid residues. The biological relevance of these sequence motifs was tested by creating a series of N-terminal deletion constructs lacking the first 35, 70, 150, and 210 amino acids, which successively remove the four regions of sequence similarity (Figure 5A). The four N-terminal truncation mutants were evaluated for their effects on Ssy5 activation, as assessed by Stp1 processing and growth on YPD plus MM. Similar to full-length Ptr3, the Δ35, Δ70, and Δ150 truncations resulted in properly regulated Stp1 processing (Figure 5B, compare lanes 1 and 2 and lanes 3 and 4, 5 and 6, and 7 and 8, respectively) and robust growth on YPD plus MM (Figure 5D, compare dilutions 1 and 2–4, respectively). In contrast, the Δ210 truncation resulted in a nonfunctional protein that did not support Stp1 processing and growth (Figure 5, B, lanes 9 and 10, and D, dilution 5). Apparently, the fourth region of conservation, between residues 150 and 210, is essential for Ptr3 function.

A closer examination of this region revealed a relatively small but highly conserved sequence motif composed of hydrophobic amino acids between amino acids 160 and 180 (Figure 5A, middle). On the basis of the amino acid composition, we designated this sequence the LFA motif, which is predicted to fold into an amphipathic α-helix (Figure 5A, bottom). To test the biological significance of this motif, we deleted 9 amino acids (aa 167–175), including the L, F, and A residues (ΔLFA; Figure 5A, middle, red bar) in the context of a myc-tagged Ptr3. The ΔLFA mutant proteins were expressed at levels similar to those of wild-type Ptr3 (Figure 5C, compare lanes 3 and 4 and lanes 1 and 2), but the mutant protein failed to support signaling; no Stp1 processing (Figure 5C, lane 4) and no growth on YPD plus MM (Figure 5D, dilution 8) was observed. These results indicate that the LFA motif of Ptr3 is important for Ssy5 activation.

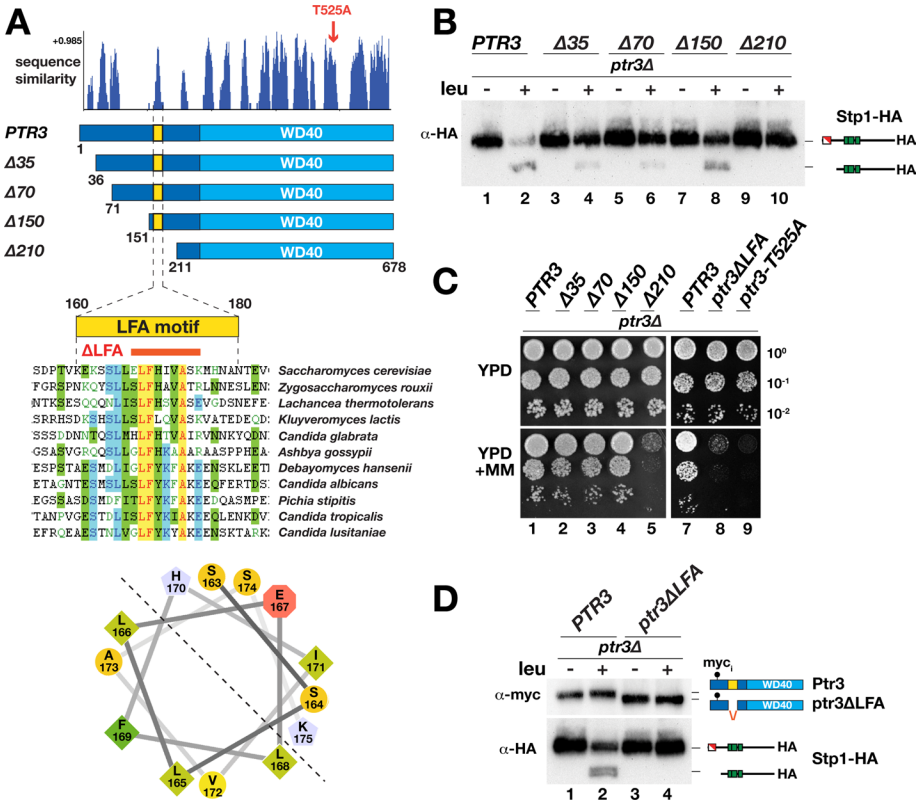

FIGURE 5: Deletion analysis of the N-terminal domain of Ptr3. (A) Similarity plot of the aligned protein sequences of Ptr3 (*S. cerevisiae*) and fungal orthologues (as in Figure 2) and schematic representations of Ptr3 and of the N-terminal deletion constructs. The positions of the N-terminal residue, each preceded by an initiator methionine, are indicated. The region between residues 160 and 180 contains sequences designated the LFA motif (yellow box) with nine core amino acids (aa 167–175). The sequence alignment of the LFA motif is shown expanded; identical (yellow), conservative (blue), and similar (green) residues are highlighted, and residues with weak (green text) or no similarity (black) are indicated. The LFA motif is predicted to form an amphipathic α-helix (Garnier *et al.*, 1996); bottom, helical wheel projection of residues 163–175. (B) Immunoblot analysis of extracts from HKY31 (*ptr3*Δ) carrying plasmids pCA122 (*STP1-HA*) and pHK017 (*PTR3*), pDO131 (*Δ35*), pDO132 (*Δ70*), pDO133 (*Δ150*), or pDO134 (*Δ210*). (C) Growth of strains (left) as in B and (right) of strain HKY31 (*ptr3*Δ) carrying plasmids pCA122 (*STP1-HA*) and pHK019 (*PTR3*), pDO138 (*ptr3ΔLFA*), or pDO96 (*ptr3-T525A*) on YPD and YPD plus MM media. (D) Immunoblot analysis of extracts from HKY31 (*ptr3*Δ) carrying plasmids pCA122 (*STP1-HA*) and pHK019 (*PTR3*) or pDO138 (*ptr3ΔLFA*). Immunoreactive forms of Stp1 (full length and processed) and Ptr3 are indicated.

The LFA domain of Ptr3 suffices to mediate the interaction with Ssy5

To test whether the LFA domain, defined as amino acids 151–210, enables Ptr3 to physically associate with Ssy5, we used a split ubiquitin approach. We fused N_{Ub} to Ptr3 (*N_{ub}-PTR3*), the first 210 amino acids of Ptr3 (*N_{Ub}-ptr3ΔCT*), and Ptr3 lacking the LFA motif (*N_{Ub}-ptr3ΔLFA*). These constructs were individually coexpressed with *SSY5-C_{Ub}-GST-HA*, and interactions were scored by the presence of the cleaved reporter (GST-HA; Figure 6A). The N_{Ub} constructs containing either full-length Ptr3 or the C-terminal deletion, both with an intact LFA domain, interacted with Ssy5 in a constitutive manner; the cleaved GST-HA reporter was detected independent of amino acid induction (Figure 6A, lanes 3 and 4 and lanes 7 and 8, respectively). Identical to N_{Ub} alone (vc), the N_{Ub} construct lacking the LFA motif failed to interact with Ssy5, and no cleaved reporter was detected (Figure 6A, lanes 1 and 2 and lanes 5 and 6, respectively). The data clearly demonstrate that an intact LFA domain is required to facilitate interactions between Ptr3 and Ssy5.

We posited that the loss of function resulting from the deletion of the LFA motif (Figure 5, C and D) could be suppressed in the context of the Ssy5–Ptr3 fusion protein (Figure 3B). We constructed and expressed an *SSY5-PTR3ΔLFA* chimera lacking the LFA motif in *ptr3*Δ *ssy5*Δ cells and found that it supported Stp1 processing and growth on YPD plus MM, as well as *SSY5-PTR3* (Figure 6B, compare lanes 1–4 and dilutions 3 and 1). In contrast, the loss of function resulting from the T525A mutation within the WD40-like domain, which perturbs the ability of Ptr3 to engage in self-interactions (Figure 2C), was not suppressed by the forced Ssy5–Ptr3 interaction; the chimeric protein carrying the T525A mutation encoded by the *SSY5-ptr3* allele did not facilitate Stp1 processing and thus failed to support growth on YPD+MM (Figure 6B, lanes 5 and 6 and dilution 2). These results indicate that the LFA domain is important for mediating interactions with Ssy5, and that Ptr3 homo-oligomerization, via its WD40-like domain, is critically required for Ssy5 activation.

Finally, to unambiguously test the role of the LFA domain of Ptr3 as the binding site for Ssy5, we assessed whether the LFA domain fused to Yck1 would suffice to direct the kinase activity toward the Ssy5 prodomain. Strikingly, amino acids 151–210 harboring the complete LFA domain fused to Yck1 (*YCK1-PTR3$_{(151-210)}$*) triggered constitutive Ssy5 activation, leading to efficient Stp1 processing (Figure 7C, lanes 6–8) and robust growth on YPD plus MM (Figure 7B, dilutions 4 and 5). In contrast, deletion of the 9 core amino acids of the LFA motif (aa 167–175), in the context of otherwise full-length Ptr3 (*YCK1-ptr3ΔLFA*) or in the isolated LFA domain (*YCK1-PTR3$_{(151-210ΔLFA)}$*), did not support growth on YPD plus MM (Figure 7B, dilutions 3 and 7, respectively). The lack of growth reflected the lack of Stp1 processing (Figure 7, C, lanes 4 and 5, and D, lanes 3 and 4, respectively). Theses results confirm that the LFA domain of Ptr3 possesses the necessary structural information to support interactions with Ssy5.

DISCUSSION

We defined the signaling events within the SPS sensor that couple the amino acid–stabilized signaling conformation of the Ssy1 receptor to the phosphorylation-dependent degradation of the Ssy5 prodomain. Prodomain degradation is the first irreversible and thus committing step in the cellular response to extracellular amino acids. Specifically our results illuminate the mechanisms governing the phosphorylation status of the Ssy5 prodomain. We show that phosphorylation is tuned through interactions of Ssy5 with the general and constitutively active Rts1-PP2A and the pathway specific factor Ptr3. Rts1 associates with Ssy5 and directs PP2A to maintain the stable hypophosphorylated inhibitory state of the prodomain in the absence of bona fide amino acid induction. Hence the constitutive

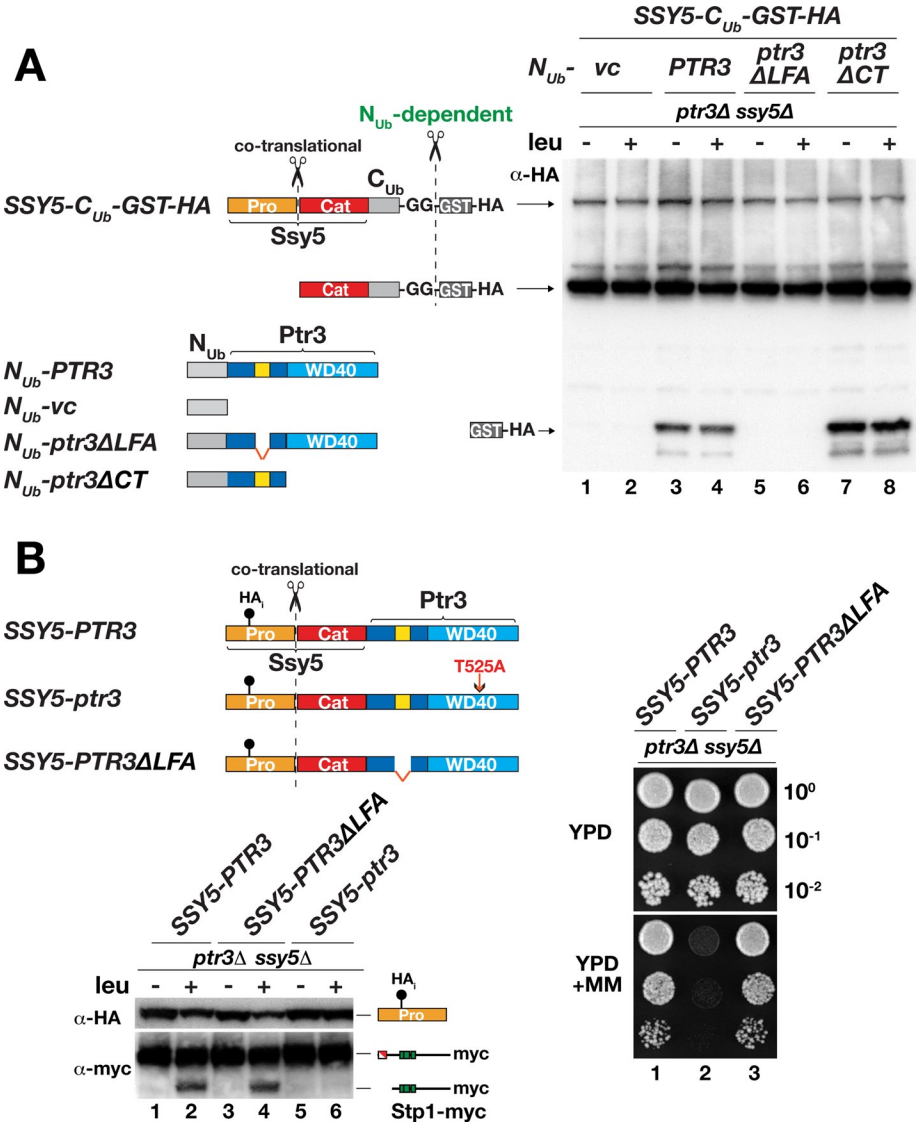

FIGURE 6: The LFA domain of Ptr3 is necessary and sufficient to mediate Ssy5 interactions. (A) Schematic diagram of the Ssy5 (C_{Ub}) and Ptr3 (N_{Ub}) fusion proteins. Scissors indicate the sites of autolytic processing of Ssy5 (cotranslational) and diagnostic cleavage indicative of split-ubiquitin interactions (N_{Ub} dependent). Immunoblot analysis of extracts from HKY85 (*ptr3Δ ssy5Δ*) carrying plasmids pDO85 (*SSY5-C$_{Ub}$-GST-HA*) and pDO88 (*N$_{Ub}$*), pDO61 (*N$_{Ub}$-PTR3*), pDO147 (*N$_{Ub}$-ptr3ΔLFA*), or pDO174 (*N$_{Ub}$-ptr3ΔCT*). Immunoreactive species generated from the Ssy5-C_{Ub}-GST-HA construct are indicated at their corresponding positions of migration. (B) Schematic representation of the Ssy5–Ptr3 fusion proteins with wild-type and mutant forms of Ptr3 fused to the C-terminus of Ssy5. Immunoblot and growth analysis on YPD and YPD plus MM media of HKY85 (*ptr3Δ ssy5Δ*) carrying plasmids pCA204 (*STP1-MYC*) and pDO79 (*SSY5-PTR3*), pDO151 (*SSY5-ptr3*), or pDO139 (*SSY5-PTR3ΔLFA*). Immunoreactive forms of Stp1 (full length and processed) and of the Ssy5 prodomain are indicated.

association of Rts1-PP2A sets a threshold for SPS-sensor signaling (Figure 1). Ptr3 possesses two discrete functional domains: an N-terminal LFA-domain that provides the basis for constitutive interactions with Ssy5 (Figures 5–7), and a C-terminal WD40-like domain required for Ptr3 oligomerization (Figure 2). These properties enable Ptr3 to function as an adapter, which in response to Ssy1 signaling presents the phosphodegron of the Ssy5 prodomain to the plasma membrane–localized Yck1/2 kinases. Thus Ptr3 facilitates prodomain phosphorylation in a manner that overrides the antagonizing phosphatase activity of Rts1-PP2A (Figures 3 and 4). Consistently, the forced interaction of Yck1 with Ptr3 is sufficient to activate Ssy5 independent of Ssy1 (Figures 4 and 7). Taken together, our findings indicate that the SPS sensor converts an extracellular stimulus into a highly specific and switch-like transcriptional response by governing the spatial proximity of Yck1/2 kinases to the Ssy5 prodomain (Figure 8).

The Ssy1 receptor is a unique member of the amino acid permease family of proteins that does not catalyze amino acid uptake (Didion *et al.*, 1998; Iraqui *et al.*, 1999; Klasson *et al.*, 1999). However, Ssy1 undergoes transporter-like conformational changes between an outward-facing (signaling) and an inward-facing (nonsignaling) conformation (Wu *et al.*, 2006). In contrast to functional transporters, amino acid binding to a single substrate-binding site imposes a reaction barrier that inhibits the conversion between these conformations (Wu *et al.*, 2006). Consequently, Ssy1 signaling is sensitive to both external and internal levels of amino acids; the SPS sensor only induces gene expression when the levels of external amino acids are higher than the levels of free amino acids in cytoplasmic pools, that is, conditions that stabilize the outward-facing conformation. Of note, in its amino acid–free state, the Ssy1 receptor is able to switch between its two conformations. Hence, even under conditions in which no stimulus is provided, a population of Ssy1 will transiently adopt the signaling conformation and can inappropriately initiate downstream signaling events. Consistent with this notion, in the absence of amino acid induction, cells lacking a functional PP2A exhibit constitutively induced SPS-sensor–regulated gene expression (Figure 1, C and D), the consequence of reduced steady-state levels of prodomain (Figure 1A). Accordingly, phosphorylated species of prodomain accumulate in *rts1Δ* cells, when prodomain degradation is blocked (Figure 1B). Thus constitutive Rts1-mediated dephosphorylation antagonizes prodomain phosphorylation and is essential to set the threshold for SPS-sensor activation required to prevent spurious signaling when the Ssy1 receptor transiently adopts the signaling conformation.

The negative control exerted by Rts1-PP2A on prodomain phosphorylation is important for two reasons. First, the subsequent SPS signaling events involve proteolytic events that are not reversible, that is, prodomain degradation and the endoproteolytic activation of Stp1/Stp2. Second, SPS-sensor signaling is amplified since the endoproteolytic activity of the Ssy5 is catalytic (Andréasson *et al.*, 2006). Consequently, a small proportion of prodomain degradation can suffice to generate significant amounts of processed forms of Stp1 and Stp2 (Andréasson and Ljungdahl, 2002). Clearly, given the sensitivity of SPS signaling, the degradation of the Ssy5 prodomain, and thus its phosphorylation status, needs to be tightly controlled.

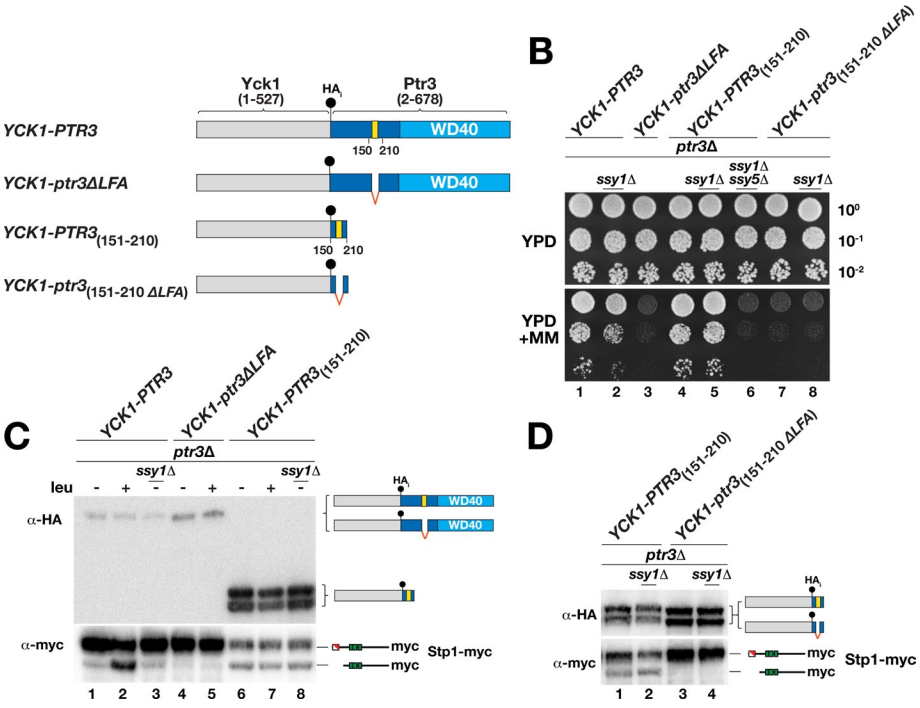

FIGURE 7: Fusion of Yck1 to the LFA domain constitutively activates Ssy5. (A) Schematic representation of the Yck1–Ptr3 fusion proteins with wild-type and mutant forms of Ptr3 fused to the C-terminus of Yck1. (B) Growth of HKY31 (*ptr3*Δ; dilutions 1, 3 and 4, and 7), HKY33 (*ptr3*Δ *ssy1*Δ; dilutions 2, 5, and 8) and CAY285 (*ssy1*Δ *ptr3*Δ*ssy5*Δ; dilution 6) carrying plasmids pCA204 (*STP1-MYC*) and pDO84 (*YCK1-PTR3*), pDO145 (*YCK1-ptr3*Δ*LFA*), pDO176 (*YCK1-PTR3*$_{151-210}$), or pDO177 (*YCK1-ptr3*$_{151-210\Delta LFA}$) on YPD and YPD plus MM media. (C) Immunoblot analysis of extracts from HKY31 (*ptr3*Δ; lanes 1 and 2 and lanes 4–7) and HKY33 (*ptr3*Δ *ssy1*Δ; lanes 3 and 8) carrying plasmid pCA204 (*STP1-MYC*) and pDO84 (*YCK1-PTR3*), pDO145 (*YCK1-ptr3*ΔLFA), or pDO176 (*YCK1-PTR3*$_{151-210}$). (D) Immunoblot analysis of extracts from HKY31 (*ptr3*Δ; lanes 1 and 3) and HKY33 (*ptr3*Δ *ssy1*Δ; lanes 2 and 4) carrying plasmid pCA204 (*STP1-MYC*) and pDO176 (*YCK1-PTR3*$_{151-210}$) or pDO177 (*YCK1-ptr3*$_{151-210\Delta LFA}$). Immunoreactive forms of Yck1–Ptr3 chimeras and of Stp1 (full length and processed) are indicated at their corresponding positions of migration.

Several findings indicate that Ptr3 functions as an adapter protein that couples conformational information reflecting signal perception by Ssy1 to the degradation of the Ssy5 prodomain, a requisite for Stp1/Stp2 processing. We confirmed that Ptr3 interacts with the N-terminal cytoplasmic domain of Ssy1 (Figure 2C; Liu *et al.*, 2008) and found that an LFA domain localized in its N-terminus facilitates constitutive interactions with Ssy5, forming a Ptr3–Ssy5 subcomplex (Figures 5–7). The active role of Ptr3 in SPS-sensor signaling is supported by our analysis of the mutant Ssy5-E131A, which exhibits constitutive activity independent of Ssy1 but retains an absolute requirement for Ptr3 and Yck1/2 (Figure 3C). Thus Ssy5-E131A adopts a conformation that facilitates Ptr3-dependent recruitment of Yck1/2. The facts that Ssy5-E131A bypasses the requirement of Ssy1 and that Ptr3 is itself inducibly phosphorylated upon amino acid stimulation, a process that occurs independently of Ssy5 (Liu *et al.*, 2008), indicates that Ptr3, and not Ssy1 or Ssy5, provides the interaction surface for Yck1/2. Taken together, these findings suggest that upon amino acid induction, when the signaling conformation of Ptr3 is stabilized via interactions with the amino acid–bound, outwardly oriented conformation of the Ssy1 receptor, Yck1/2 gains extended access to the Ptr3–Ssy5 subcomplex. As a consequence, the constitutive activity of Rts1-PP2A is overcome, resulting in hyperphosphorylation of Ptr3 and, crucially, of the Ssy5 prodomain.

Our study demonstrates how a dedicated adapter protein facilitates the regulated activation of an intracellular signaling protease in response to conformational states of a plasma membrane–localized receptor. It is intriguing that the mechanisms governing SPS-sensor signaling exhibit striking similarity to those controlling NFκB activation. NFκB transcription factors are retained in the cytoplasm by association with inhibitory IκB proteins. Stimulus-dependent IκB phosphorylation leads to its ubiquitylation and subsequent proteasomal degradation, enabling translocation of NFκB to the nucleus (Karin, 1999). Like Ptr3 in the activation of Ssy5, NEMO functions as an adapter that facilitates IκB phosphorylation by recruiting the catalytic IκB kinase components of IKKα and IKK*β* (Schrofelbauer *et al.*, 2012). It has been reported that induced oligomerization of NEMO, IKKα, or IKK*β* leads to activation of NFκB (Inohara *et al.*, 2000; Poyet *et al.*, 2000). Oligomerization of NEMO may induce oligomerization of the IKKα/β kinase and, consequently, induction of its activity (Poyet *et al.*, 2000). Similarly, as described here, we found that Ptr3 multimerizes via its WD40-like domain and that homo-oligomerization is essential for its role in Ssy5 activation (Figure 2C); the nonfunctional ptr3-T525A carrying a mutation affecting the WD40-like domain does not oligomerize but retains the ability to interact with the N-terminal domain of Ssy1 (Figure 2C) and Ssy5 (Figure 3A). Presumably, oligomerization of Ptr3 is required for the amino acid–induced juxtaposition of Yck1/2 and Ssy5 prodomain. The concept of the requirement of protein multimerization to facilitate signal transduction has been well established, and examples include signaling induced by G protein–coupled receptors (Lee *et al.*, 2003; Maggio *et al.*, 2007) and receptor tyrosine kinases (Ullrich and Schlessinger, 1990; Lemmon and Schlessinger, 2010). Furthermore, the significance and implications of the observed ability of the scaffold protein Ste5 to multimerize is under discussion in studies focused on mitogen-activated protein kinase signaling (Yablonski *et al.*, 1996; Wang and Elion, 2003; Lamson *et al.*, 2006).

MATERIALS AND METHODS

Yeast strains and plasmids

The *S. cerevisiae* strains and plasmids used in this work are listed in Tables 1 and 2, respectively. The yeast strains are isogenic descendants of the S288C-derived strain AA255/PLY115 (Antebi and Fink, 1992) or of BY4741 and BY4742, with the exception of the two-hybrid strain AH109. Strain AH109 is a derivative of strain PJ69-2A and includes the *ADE2* and *HIS3* markers (James *et al.*, 1996). The sequences of mutagenic oligonucleotides and PCR primers for homologous recombination are available upon request.

Media

Standard media, including YPD medium, ammonia-based synthetic minimal dextrose (SD) medium, supplemented as required to

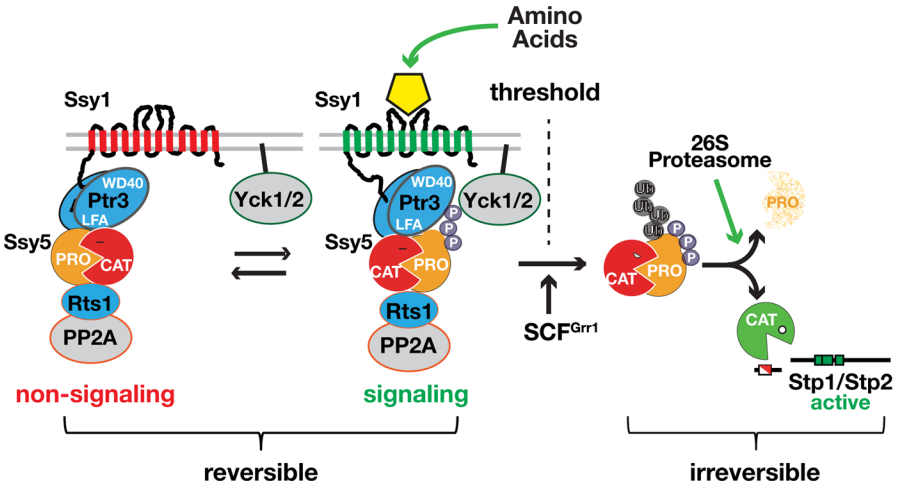

FIGURE 8: Model of the mechanisms controlling Ssy5 activation. In the absence of amino acid induction, the Ssy1 receptor is able to reversibly switch between nonsignaling and signaling conformations. The constitutive activity of Rts1-PP2A sets a signaling threshold by maintaining the prodomain in its stable hypophosphorylated state in the absence of extracellular amino acids. The signaling conformation of Ssy1 is stabilized by the binding of an extracellular amino acid. In the context of the signaling conformation, Ptr3 facilitates the juxtaposition of Ssy5 and Yck1/2 kinases, which favors prodomain phosphorylation. Prodomain phosphorylation is critical for the subsequent irreversible signaling events, that is, prodomain degradation and endoproteolytic processing of Stp1/Stp2.

enable growth of auxotrophic strains, and ammonia-based synthetic complete dextrose (SC) were prepared as described (Andréasson and Ljungdahl, 2002). Sensitivity to MM (100 µg/ml) was monitored on YPD medium as described (Jørgensen *et al.*, 1998; Andréasson and Ljungdahl, 2002). Briefly, 10-fold dilutions of cultures were spotted on YPD and YPD plus MM media. Plates were incubated at 30°C for 2–3 d and photographed.

Immunoblot analysis

Whole-cell extracts were prepared under denaturing conditions using NaOH and trichloroacetic acid as described previously (Silve *et al.*, 1991). Cells were grown in SD media (–) and, when indicated (+), L-leucine (leu) was added at a concentration of 1.3 mM for 30 min to induce SPS-sensor signaling before extract preparation. Primary antibodies were diluted as follows: 3F10 anti–HA–horseradish peroxidase (HRP; Roche Applied Science, Indianapolis, IN), 1:2000; anti–myc-HRP 9E10 monoclonal antibody (Roche Applied Science), 1:2000; and anti-Pgk1 (Molecular Probe, Eugene, OR), 1:10,000. Immunoreactive bands were visualized by chemiluminescence detection (SuperSignal West Dura Extended-Duration Substrate; Pierce, Rockford, IL) and quantified using a LAS1000 system (Fuji Photo Film, Tokyo, Japan).

Directed yeast two-hybrid assay

Two-hybrid interactions between Gal4 DBD and Gal4 AD fusion proteins were tested in strain AH109. Growth of transformants on SC media lacking tryptophan and leucine (nonselective) and SC media lacking tryptophan, leucine, histidine, and adenine (selective)

Strain	Genotype	Reference/source
AH109	*MATa trp1-901 leu2-3, 112 ura3-52 his3-200, gal4Δ gal80Δ LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3 GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2 URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*	James *et al.* (1996), A. Holtz (unpublished data)
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
BY4741 *rts1Δ*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rts1Δ::kanMX4*	EUROSCARF
BY4741 *reg1Δ*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 reg1Δ::kanMX*	ResGen/Invitrogen (Carlsbad, CA)
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	ResGen/Invitrogen
CAY272	*MATa lys2Δ201 ura3-52 ptr3Δ15::hisG grr1Δ50::hphMX4*	Ljungdahl laboratory
CAY285	*MATa lys2Δ201 ura3-52 ptr3Δ15::hisG ssy1Δ13::hisG ssy5Δ2::hisG*	Pfirrmann *et al.* (2010)
CAY307	*MATa lys2Δ201 ura3-52 ptr3Δ15::hisG ssy5Δ2::hisG grr1Δ50::hphMX4*	Ljungdahl laboratory
DC152 (BY4742 *pph21Δ pph22Δ*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pph21Δ::KanMX pph22Δ::kanMX*	Castermans *et al.* (2012)
DOY03 (BY4741 *grr1Δ*)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 grr1Δ::natMX*	This study
DOY04 (BY4741 *rts1Δ grr1Δ*)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rts1Δ::kanMX4 grr1Δ:: natMX*	This study
HKY31	*MATa lys2Δ201 ura3-52 52 ptr3Δ15::hisG*	Klasson *et al.* (1999)
HKY33	*MATa lys2Δ201 ura3-52 ssy1Δ13::hisG ptr3Δ15::hisG*	Klasson *et al.* (1999)
HKY85	*MATa lys2Δ201 ura3-52 ptr3Δ15::hisG ssy5Δ2::hisG*	Forsberg and Ljungdahl (2001)

EUROSCARF, European *Saccharomyces cerevisiae* Archive for Functional Analysis, Institute for Molecular Biosciences, Johann Wolfgang Goethe-University Frankfurt, Frankfurt, Germany.

TABLE 1: Yeast strains used in this study.

Plasmid	Description	Reference/source
pAB1	pRS313 carrying *HIS3*, *MET15*, and *LEU2*	This study
pACTII	Yeast two-hybrid plasmid (*LEU2*) carrying Gal4 activation domain	Clontech (Mountain View, CA)
pACTII-Ssy5	pACTII (*LEU2*) carrying the *SSY5* ORF	Liu *et al.* (2008)
pACTII-Ptr3	pACTII (*LEU2*) carrying the *PTR3* ORF	Liu *et al.* (2008)
pAGP1-lacZ	YCpAGP1-lacZ (*URA3*)	Iraqui *et al.* (1999)
pCA122	pRS317 (*LYS2*) containing *STP1-3HA*	Andréasson and Ljungdahl (2004)
pCA204	pRS317 (*LYS2*) containing *STP1-MYC-kanMX*	Andréasson *et al.* (2006)
pCJ353	YCp-*SSY5-42-HA6* (*URA3)*	Abdel-Sater *et al.* (2011)
pDO61	pRS316 (*URA3*) containing N_{Ub}-*PTR3*	This study
pDO79	pRS316 (*URA3*) containing HA_i-*SSY5-PTR3*	This study
pDO84	pRS316 (*URA3*) containing $YCK1_{2\text{-}527}$-HA_i-*PTR3*	This study
pDO85	pRS317 (*LYS2*) containing *SSY5*-C_{ub}-*GST-HA*	This study
pDO86	pRS316 (*URA3*) containing $yck1_{2\text{-}527}$-*K98R*-HA_i-*PTR3*	This study
pDO88	pRS316 (*URA3*) containing N_{Ub}	This study
pDO94	pRS316 (*URA3*) containing N_{Ub}-*ptr3-T525A*	This study
pDO95	pRS316 (*URA3*) containing $YCK1_{2\text{-}527}$-HA_i-*PTR3-T525A*	This study
pDO96	pRS316 (*URA3*) MYC_i-*ptr3-T525A*	This study
pDO98	pRS316 (*URA3*) containing $yck1_{2\text{-}527}$-*K98R*-HA_i-*ptr3-T525A*	This study
pDO131	pRS316 (*URA3*) containing *PTR3Δ35*	This study
pDO132	pRS316 (*URA3*) containing *PTR3Δ70*	This study
pDO133	pRS316 (*URA3*) containing *PTR3Δ150*	This study
pDO134	pRS316 (*URA3*) containing *ptr3Δ210*	This study
pDO138	pRS316 (*URA3*) MYC_i-*ptr3ΔLFA*	This study
pDO139	pRS316 (*URA3*) containing HA_i-*SSY5-PTR3*ΔLFA	This study
pDO145	pRS316 (*URA3*) containing $YCK1_{2\text{-}527}$-HA_i-*ptr3*ΔLFA	This study
pDO147	pRS316 (*URA3*) containing N_{Ub}-*ptr3ΔLFA*	This study
pDO148	pRS317 (*LYS2*) containing *GST-HA-PTR3*	This study
pDO149	pRS317 (*LYS2*) containing *GST-HA-PTR3-T525A*	This study
pDO151	pRS316 (*URA3*) containing HA_i-*SSY5-ptr3*-T525A	This study
pDO162	pRS316 (*URA3*) containing $YCK1_{2\text{-}527}$-HA_i-$PTR3_{1\text{-}210}ΔCT$	This study
pDO174	pRS316 (*URA3*) containing N_{Ub}-$PTR3_{1\text{-}210}ΔCT$	This study
pDO176	pRS316 (*URA3*) containing $YCK1_{2\text{-}527}$-HA_i-$PTR3_{151\text{-}210}$	This study
pDO177	pRS316 (*URA3*) containing $YCK1_{2\text{-}527}$-HA_i-$ptr3_{151\text{-}210}Δ_{LFA}$	This study
pDO181	pRS317 (*LYS2*) containing $ssy5_{Cat}$-C_{Ub}-*GST-HA* (Cat = aa 382-699)	This study
pDO182	pRS317 (*LYS2*) containing $ssy5_{Pro}$-C_{Ub}-*GST-HA* Pro (Pro = aa 1–381)	This study
pDO185	pGBKT7 (*TRP1*) carrying *RTS1* ORF	This study
pDO186	pGBKT7 (*TRP1*) carrying *PTR3* ORF	This study
pDO189	pACTII (*LEU2*) carrying *ptr3-T525A*	This study
pGBKT7	Yeast two-hybrid plasmid (*TRP1*) carrying Gal4 DNA-binding domain	Clontech
pGBKT7-SSY1N	pGBKT7 (*TRP1*) carrying sequence encoding N-terminal amino acid residues 2–273 of Ssy1	Liu *et al.* (2008)
pHK017	pRS316 (*URA3*) containing *PTR3*	Klasson *et al.* (1999)
pHK019	pRS316 (*URA3*) MYC_i-*PTR3* (3× myc, between aa 157 and 158)	Ljungdahl laboratory
pHK048	pRS316 (*URA3*) containing *MYC-SSY5*	Forsberg and Ljungdahl (2001)
pRS316	pRS316 (*URA3*)	Sikorski and Hieter (1989)
pRS317	pRS317 (*LYS2)*	Sikorski and Hieter (1989)
pSH120	pRS316 (*URA3*) containing HA_i-*SSY5-GST*	Pfirrmann *et al.* (2010)
pTP115	pRS316 (*URA3*) containing HA_i-*SSY5-E131A-GST*	Pfirrmann *et al.* (2010)

ORF, open reading frame.

TABLE 2: Plasmids used in this study.

indicating interactions was assessed. Tenfold dilutions of cultures were spotted on nonselective and selective media, and plates were photographed 2–4 d after incubation at 30°C.

Split ubiquitin protein–protein interaction assay

The split ubiquitin protein–protein interaction assay is based on two findings: ubiquitin can be divided into two halves that spontaneously reassemble when coexpressed (Johnsson and Varshavsky, 1994), and ubiquitin that is fused to proteins is rapidly cleaved off by ubiquitin-specific proteases (Bachmair *et al.*, 1986). These properties can be experimentally exploited to detect protein–protein interactions by creating proteins fused to either half of ubiquitin; when the C-terminal fragment of ubiquitin (C_{Ub}) is expressed as a fusion with an immunologically detectable reporter protein (e.g., GST-HA), the reporter protein is cleaved away if the N-terminal fragment of ubiquitin (N_{Ub}) is presented in a context that brings it into close proximity of the C_{Ub} fragment. The stringency of the assay is greatly enhanced if a variant of N_{Ub} carrying an alanine instead of isoleucine at position 13 is used; the I13A mutant N_{Ub} exhibits significantly decreased spontaneous association with C_{Ub} (Johnsson and Varshavsky, 1994). Immunoblot analysis of extracts prepared from strains coexpressing N_{Ub} and C_{Ub} constructs was performed; the detection of the cleaved-off GST-HA reporter protein reflects an interaction between the two proteins of interest.

β-Galactosidase activity assay

The β-gal activity was determined with *N*-lauroyl-sarcosine–permeabilized cells (Kippert, 1995). Semiquantitative measurements of β-galactosidase activity used equally turbid cell suspensions (OD_{600} = 1) diluted 1:1 in 0.4 M potassium phosphate buffer (pH 7) containing 0.2% (wt/vol) Na *N*-lauroyl-sarcosine and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). Cell suspensions were incubated at 30°C.

ACKNOWLEDGMENTS

We thank Thorsten Pfirrmann and the members of the Andréasson, Ott, and Ljungdahl laboratories for constructive comments throughout the course of this work. We acknowledge Anna Schick and Hanna Forsberg for constructing plasmids and thank Bruno André (Université Libre de Bruxelles, Belgium) and Zhengchang Liu (University of New Orleans, New Orleans, LA) for plasmids and Claes Andréasson and Johan Thevelein (KU Leuven, Leuven, Belgium) for yeast strains. This research was supported by funding from the Swedish Research Council (P.O.L.).

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