

Specific α -Arrestins Negatively Regulate *Saccharomyces cerevisiae* Pheromone Response by Down-Modulating the G-Protein-Coupled Receptor Ste2

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G-protein-coupled receptors (GPCRs) are integral membrane proteins that initiate responses to extracellular stimuli by mediating ligand-dependent activation of cognate heterotrimeric G proteins. In yeast, occupancy of GPCR Ste2 by peptide pheromone α -factor initiates signaling by releasing a stimulatory G $\beta\gamma$ complex (Ste4-Ste18) from its inhibitory G α subunit (Gpa1). Prolonged pathway stimulation is detrimental, and feedback mechanisms have evolved that act at the receptor level to limit the duration of signaling and stimulate recovery from pheromone-induced G_i arrest, including upregulation of the expression of an α -factor-degrading protease (Bar1), a regulator of G-protein signaling protein (Sst2) that stimulates Gpa1-GTP hydrolysis, and Gpa1 itself. Ste2 is also downregulated by endocytosis, both constitutive and ligand induced. Ste2 internalization requires its phosphorylation and subsequent ubiquitinylation by membrane-localized protein kinases (Yck1 and Yck2) and a ubiquitin ligase (Rsp5). Here, we demonstrate that three different members of the α -arrestin family (Ldb19/Art1, Rod1/Art4, and Rog3/Art7) contribute to Ste2 desensitization and internalization, and they do so by discrete mechanisms. We provide genetic and biochemical evidence that Ldb19 and Rod1 recruit Rsp5 to Ste2 via PPXY motifs in their C-terminal regions; in contrast, the arrestin fold domain at the N terminus of Rog3 is sufficient to promote adaptation. Finally, we show that Rod1 function requires calcineurin-dependent dephosphorylation.

For survival, eukaryotic cells sense and respond to changes in external conditions using, in many cases, a G-protein-coupled receptor (GPCR) (1, 2). Most clinically used pharmaceuticals act on GPCRs (3, 4). After an initial response, cells normally adapt by becoming desensitized to the stimulus, whereas chronic GPCR action can lead to inflammation and other pathophysiology (5, 6). Thus, the mechanisms underlying both GPCR signal transmission and its attenuation have important medical implications.

In *Saccharomyces cerevisiae*, GPCR Ste2 in the plasma membrane (PM) of a *MATa* haploid cell binds α -factor (a 13-residue peptide) secreted by a *MAT α* haploid cell, and a *MAT α* haploid uses GPCR Ste3 to bind α -factor (a 12-residue prenylated peptide) released by a *MATa* haploid (7). Engagement of these receptors by these ligands (mating pheromones) initiates a mitogen-activated protein kinase (MAPK) cascade. The activated MAPK evokes transcriptional and morphological responses that arrest cell growth in the G₁ phase of the cell cycle and convert the cells to gametes, which conjugate (mate) to form a *MATa*/*MAT α* diploid (8, 9). Genetic and molecular analysis of this system has established many important concepts in GPCR-initiated signaling and its regulation (10–13).

Hyperactivation or prolonged signaling through the mating pheromone response pathway can cause cell death (14). To avoid this, α -factor also induces feedback mechanisms that downregulate signaling in *MATa* cells. Paramount among these adaptation processes is upregulation of expression of genes for a variety of negative regulators, such as *MSG5*, which encodes a phosphoprotein phosphatase that deactivates the terminal MAPK (Fus3) of the pathway (15, 16). However, induced negative regulators also include factors that desensitize the pathway far upstream by acting

on α -factor, its receptor, or the associated heterotrimeric G protein, preventing further GPCR-initiated signaling. For example, *BAR1* encodes a protease that cleaves α -factor into two inactive fragments (17, 18). *SST2* encodes the first regulator of G-protein signaling (RGS) identified (7, 19, 20). Binding of its N-terminal DEP domains to the cytosolic tail of Ste2 (21) delivers Sst2 to the PM. Thus, its C-terminal RGS domain is positioned to stimulate conversion of PM-localized GTP-bound Gpa1 back to its GDP state (22). GDP-Gpa1 then reassociates with and blocks downstream signaling by the Ste4-Ste18 complex (23, 24), which is also PM anchored via S-palmitoylation and S-farnesylation of Ste18 (25, 26). Recoupling and squelching of G $\beta\gamma$ function is further promoted by mass action, because *GPA1* is upregulated in response to pheromone (27), concomitant with an enhanced rate of its N-myristoylation (28), a posttranslational modification essential for Gpa1 PM targeting, coupling to G $\beta\gamma$, and association with the receptor (23, 29).

Termination of an agonist-initiated GPCR-mediated signal is also thought to involve ligand-induced receptor endocytosis (30,

Received 15 February 2014 Returned for modification 11 March 2014

Accepted 1 May 2014

Published ahead of print 12 May 2014

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doi:10.1128/MCB.00230-14

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31). It has been known for decades that both Ste2 and Ste3 undergo constitutive internalization at a basal rate and that endocytosis is stimulated upon binding of the cognate pheromone (32–36). Pheromone binding causes a conformational change that promotes phosphorylation of the Ste2 and Ste3 C termini (37–39), mediated by PM-anchored casein kinase I isoforms (Yck1 and Yck2) (21, 40). Phosphorylation is a prelude to receptor ubiquitinylation (39, 41) by a PM-associated HECT domain-containing ubiquitin ligase (E3), Rsp5 (42–44) (the mammalian ortholog is NEDD4L [45]). Ubiquitin attachment targets the receptors for clathrin-mediated internalization (46, 47). The resulting endosomes are delivered to the vacuole (equivalent to a mammalian lysosome) where the receptor is degraded (48, 49).

Subsequent studies have provided more detailed analysis of spatiotemporal aspects of Ste2 (50, 51) and Ste3 (52, 53) internalization and better delineated the phosphorylation and ubiquitinylation sites (54, 55). However, how Rsp5 recognizes and is recruited to these GPCRs to catalyze ubiquitinylation remained unresolved. Discovery in yeast of a protein family, called the α -arrestins, that serve as adaptors for Rsp5-dependent ubiquitinylation and internalization of nutrient permeases provided a clue (56–58). The *S. cerevisiae* genome encodes 14 currently recognized members of the α -arrestin family (57–60). The name derives from homology of their N-terminal domains to a fold found in mammalian arrestin and β -arrestins (56, 61), first identified and implicated in blocking signaling by rhodopsin (62) and the β -adrenergic receptor (63), respectively.

As described here, we found that three different α -arrestins, Ldb19/Art1, Rod1/Art4, and Rog3/Art7, have overlapping functions and contribute to Ste2 internalization and MATa cell recovery from pheromone-induced G₁ arrest. Surprisingly, the modes of action of these α -arrestins are distinct. Ldb19 plays a role primarily in basal turnover of Ste2, whereas Rod1 and Rog3 contribute to desensitization of the agonist-occupied receptor. Although paralogous on the basis of primary sequence, Rod1 and Rog3 act by different mechanisms; Rod1 is obligatorily Rsp5 dependent, but Rog3 is not. Moreover, Rod1 (but not Rog3) function during Ste2 downregulation requires calcineurin (CN)-dependent dephosphorylation. Together, our studies extend the function of α -arrestins to another class of polytopic membrane protein and demonstrate discrete actions of α -arrestin family members whose functions previously have been enigmatic.

MATERIALS AND METHODS

Strains and growth conditions. Yeast strains (Table 1) were grown at 30°C in either rich (YPD) or synthetic complete (SC) medium containing 2% glucose (unless another carbon source is specified) and with appropriate nutrients to maintain selection for plasmids, if present (64). Standard genetic methods were used for strain construction (65).

Plasmids. Plasmids (Table 2) were constructed using standard procedures (66). DNA amplification by PCR (66) employed Phusion DNA polymerase (New England BioLabs, Ipswich, MA), and all constructs were verified by sequencing. Site-directed mutagenesis (67) was carried out using the same DNA polymerase and QuikChange methodology (New England BioLabs) according to the manufacturer's instructions.

Pheromone-imposed growth arrest. Response to α -factor was assessed by an agar diffusion (halo) bioassay essentially as described previously (37). In brief, cells ($\sim 10^5$) were plated in top agar on solid YPD or SC medium as appropriate. On the resulting surface were laid sterile cellulose filter disks, onto which an aliquot (typically, 15 μ l) of an aqueous solution (1 mg/ml) of α -factor (GeneScript, Piscataway, NJ) had been

aseptically spotted, and the plates were incubated at 30°C for 2 to 4 days. To induce α -arrestin overexpression, strains containing the tripartite *S. cerevisiae* Gal4-human estrogen receptor-herpes simplex virus transactivator VP16 fusion protein (Gal4-ER-VP16 or GEV) (68, 69) and URA3-marked multicopy (2 μ m DNA) plasmids expressing from the *GAL1*, 10 promoter the α -arrestin of interest (as a fusion to the C terminus of glutathione S-transferase [GST]) were grown to mid-exponential phase. The cultures were then treated with β -estradiol (20 μ M final concentration) for 3 h and plated using top agar containing 200 nM β -estradiol. Samples of the same cultures were analyzed by immunoblotting (see below) to confirm α -arrestin expression.

Mating pathway activation. As one measure of pathway activation, the level of dually phosphorylated Fus3 was assessed as follows. Strains of interest carrying a *bar1* Δ mutation were grown in YPD to mid-exponential phase, a sample was removed (zero time point), and immediately thereafter the culture was treated with α -factor (15 nM final concentration). Additional samples of equivalent numbers of cells then were removed at 10, 30, 60, and 90 min, harvested by rapid sedimentation in a microcentrifuge, and immediately frozen in liquid N₂. The cell pellets were thawed on ice, and whole-cell protein extracts were prepared by alkaline lysis followed by collection of total protein by trichloroacetic acid (TCA) precipitation (70, 71). Protein precipitates were solubilized in SDS-urea gel sample buffer (5% SDS, fresh 8 M urea, 1% β -mercaptoethanol, 0.1 mM EDTA, 40 mM Tris-HCl [pH 6.8]) with 0.1% bromophenol blue, heated at 37°C for 15 min, resolved in a slab gel by SDS-PAGE (72), and analyzed by immunoblotting.

As an independent measure of pathway activation, induction of an integrated single-copy *FUS1_{prom}*-enhanced green fluorescent protein (eGFP) reporter (73) was monitored. Strains of interest carrying a *bar1* Δ mutation and also containing integrated *FUS1_{prom}*-eGFP were constructed (Table 1). These cells were grown to mid-exponential phase and treated with 15 nM α -factor for 2 h, and the level of GFP expression was quantified by flow cytometry using a Beckman-Coulter FC-500 analyzer. The resulting data were analyzed using FlowJo software (Tree Star, Inc.).

Immunoblotting. Equal numbers of cells from mid-exponential-phase cultures were collected by centrifugation and lysed, and total protein was collected and resolved by SDS-PAGE as described above. The proteins in the resulting slab gels were transferred electrophoretically to nitrocellulose sheets (74) using a semidry transfer apparatus (Transblot SD; Bio-Rad, Inc.). After blocking with carrier protein (75), the filters were incubated (generally for several h at room temperature or overnight at 4°C) with the following primary antibodies, as appropriate: rabbit polyclonal anti-GST (Sigma), rabbit polyclonal anti-phospho-extracellular signal-regulated kinase (anti-phospho-ERK; Cell Signaling), goat polyclonal anti-Fus3 (Santa Cruz), mouse monoclonal anti-HA (12CA5; Roche), mouse monoclonal antiubiquitin (P4D1; Santa Cruz), and, as loading control, rabbit polyclonal anti-Pgk1 (76). The resulting immune complexes were then detected by incubation, as appropriate, with infrared dye (IRDye 680/800)-labeled secondary antibodies, namely, goat anti-mouse IgG, goat anti-rabbit IgG, or donkey anti-goat antibody (all from Li-Cor, Lincoln, NE), followed by visualization using an infrared imager (Odyssey; Li-Cor). In experiments assessing the ratio of phosphorylated to total Fus3, band intensities were quantified for each time point using ImageJ software (National Institutes of Health). Alternatively, in some experiments, to monitor GFP-tagged proteins and after blocking with SuperBlock (Thermo Scientific, Rockford, IL), filters were incubated with rabbit polyclonal anti-GFP antibodies (Invitrogen), and the resulting immune complexes were detected with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibodies (GE Healthcare). To monitor GST-tagged proteins, the complexes were incubated with mouse monoclonal anti-GST antibodies (Covance) and detected with horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies (GE Healthcare). The bound immune complexes were then visualized by chemiluminescence using either ECL Western blotting substrate (Pierce) or SuperSignal West

TABLE 1 Yeast strains used in this study

Strain	Genotype	Reference or source
BY4741	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0</i>	Yeast deletion collection (Open Biosystems, Inc.)
<i>9arrΔ</i> (EN60)	<i>ecm21Δ::KANMX csr2Δ::KANMX bsd2Δ rog3Δ::NATMX rod1Δ ygr068cΔ aly2Δ aly1Δ ldb19Δ ylr392cΔ::HIS3 his3 leu2Δ0 ura3Δ0</i>	58
<i>ldb19Δ</i>	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 ldb19Δ::KANMX4</i>	Yeast deletion collection (Open Biosystems, Inc.)
<i>rod1Δ rog3Δ</i> (JT5858)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4</i>	This study
<i>aly1Δ aly2Δ</i> (D2-6A)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 aly1Δ::KANMX4 aly2Δ::KANMX4</i>	86
<i>ecm21Δ csr2Δ</i> (JT6751)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 ecm21Δ::KANMX4 csr2Δ::KANMX4</i>	This study
<i>art5Δ rim8Δ</i> (JT5860)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 art5Δ::KANMX4 rim8Δ::KANMX4</i>	This study
<i>art10Δ</i>	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 art10Δ::KANMX4</i>	Yeast deletion collection (Open Biosystems, Inc.)
<i>rod1Δ rog3Δ ldb19Δ</i> (JT6675)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 ldb19Δ::NATMX</i>	This study
<i>bar1Δ</i> (JT5915)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 bar1Δ::CgLEU2</i>	This study
<i>ldb19Δ bar1Δ</i> (JT5916)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 ldb19Δ::KANMX4 bar1Δ::CgLEU2</i>	This study
<i>rod1Δ rog3Δ bar1Δ</i> (JT5917)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 bar1Δ::CgLEU2</i>	This study
<i>rod1Δ rog3Δ ldb19Δ bar1Δ</i> (JT6674)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 ldb19Δ::NATMX bar1Δ::CgLEU2</i>	This study
<i>sst2Δ</i> (JT6755)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 sst2Δ::SpHIS5</i>	This study
<i>ldb19Δ sst2Δ</i> (JT6660)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 ldb19Δ::KANMX4 sst2Δ::SpHIS5</i>	This study
<i>rod1Δ rog3Δ sst2Δ</i> (JT6702)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 sst2Δ::SpHIS5</i>	This study
<i>rod1Δ rog3Δ ldb19Δ sst2Δ</i> (JT6662)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 ldb19Δ::NATMX sst2Δ::SpHIS5</i>	This study
<i>sst2Δ GEV</i> (JT5919) ^b	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 sst2Δ::SpHIS5 leu2Δ0::GEV::NATMX</i>	This study
<i>rod1Δ rog3Δ ldb19Δ sst2Δ GEV</i> (JT6716)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 ldb19Δ::NATMX sst2Δ::SpHIS5 leu2Δ0::GEV::NATMX</i>	This study
<i>STE2-mCherry bar1Δ</i> (JT6677)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-mCherry::URA3 bar1Δ::CgLEU2</i>	This study
<i>STE2-mCherry ldb19Δ bar1Δ</i> (JT6678)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-mCherry::URA3 ldb19Δ::KANMX4 bar1Δ::CgLEU2</i>	This study
<i>STE2-mCherry rod1Δ rog3Δ bar1Δ</i> (JT6679)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-mCherry::URA3 rod1Δ::KANMX4 rog3Δ::KANMX4 bar1Δ::CgLEU2</i>	This study
<i>STE2-mCherry rod1Δ rog3Δ ldb19Δ bar1Δ</i> (JT6680)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-mCherry::URA3 rod1Δ::KANMX4 rog3Δ::KANMX4 ldb19Δ::NATMX bar1Δ::CgLEU2</i>	This study
<i>STE2-GFP</i> (JT6757)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-GFP::HPH</i>	This study
<i>9arrΔ STE2-GFP</i> (JT6758)	<i>ecm21::G418 csr2::G418 bsd2 rog3::NATMX rod1 ygr068c aly2 aly1 ldb19 ylr392c::HIS3 his3 leu2Δ0 ura3Δ0 STE2-GFP::HPH</i>	This study
<i>ldb19Δ STE2-GFP</i> (JT6759)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 ldb19Δ::KANMX4 STE2-GFP::HPH</i>	This study
<i>rod1Δ rog3Δ STE2-GFP</i> (JT6760)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-GFP::HPH rod1Δ::KANMX4 rog3Δ::KANMX4</i>	This study
<i>rod1Δ rog3Δ ldb19Δ STE2-GFP</i> (JT6761)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-GFP::HPH rod1Δ::KANMX4 rog3Δ::KANMX4 ldb19Δ::NATMX</i>	This study
<i>aly1Δ aly2Δ STE2-GFP</i> (JT6762)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-GFP::HPH aly1Δ::KANMX4 aly2Δ::KANMX4</i>	This study
<i>bar1Δ FUS1_{prom}-eGFP</i> (JT6686) ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 bar1Δ::URA3 FUS1_{prom}-eGFP::LEU2</i>	This study
<i>rod1Δ rog3Δ ldb19Δ bar1Δ FUS1_{prom}-eGFP</i> (JT6668) ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 ldb19Δ::NATMX bar1Δ::URA3 FUS1_{prom}-eGFP::LEU2</i>	This study
BJ5459	<i>MATa ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4Δ::HIS3 prb1Δ1.6R can1 GAL cir⁺</i>	160
BJ5459 <i>GEV</i> (JT6743) ^b	<i>MATa ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4Δ::HIS3 prb1Δ1.6R can1 GAL leu2Δ1::GEV::NATMX</i>	This study
<i>cnb1Δ</i>	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 cnb1Δ::KANMX4</i>	Yeast deletion collection (Open Biosystems, Inc.)
<i>cna1Δ cna2Δ</i> (JT5574)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 cna1Δ::KANMX4 cna1Δ::KANMX4</i>	116
JRY11	<i>MATa ura3-52 trp1 lys2-801 leu2-Δ1 his3-Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL CNA2-S-TEV-ZZ-KANMX6 CNA1-GFP-LEU2</i>	116
<i>cnb1Δ GEV</i> (JT6694) ^b	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NATMX cnb1Δ::KANMX4</i>	This study
<i>cna1Δ cna2Δ GEV</i> (JT6695) ^b	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NATMX cna1Δ::KANMX4 cna1Δ::KANMX4</i>	This study

^a To generate strains with the integrated *FUS1_{prom}-eGFP* reporter, the cassette (73) was amplified by PCR and introduced by DNA-mediated transformation into a *bar1Δ* derivative of BY4741. The resulting strain (JT6686) was mated to a *MATa ldb19Δ rod1Δ rog3Δ bar1Δ* mutant. The resulting diploids were sporulated, and *MATa ldb19Δ rod1Δ rog3Δ ldb19Δ bar1Δ FUS1_{prom}-eGFP* spores were identified after tetrad dissection.

^b To generate a *GEV*-expressing version of the indicated yeast strain, *pACT1-GEV* (69, 161) was digested with *EcoRV* and introduced into the cells of interest by DNA-mediated transformation (65), and nourseothricin (NAT)-resistant colonies were selected in which *GEV* (expressed under the control of an *ACT1* promoter) is integrated at the *leu2Δ0* locus.

Dura extended-duration substrate (Thermo Scientific, Rockford, IL) detected with Biomax XAR film (Eastman Kodak, Rochester, NY).

Protein purification. GST-Rod1 fusions were used to assess the role of the apparent PXIXIT motif (⁵⁴⁵PQIKIE⁵⁵⁰) in this α-arrestin in mediating its association with calcineurin as follows. Cells of yeast strain JRY11,

which expresses Cna1-GFP, were transformed with pEGKG-Rod1^{WT} or pEGKG-Rod1^{AQAKAA}. The resulting transformants were grown to mid-exponential phase in SC-2% raffinose, and then expression of the α-arrestin was induced by addition of 2% galactose (final concentration). To activate calcineurin, cells were treated with 200 mM CaCl₂ (final concn-

TABLE 2 Plasmids used in this study

Plasmid	Genotype	Description/ reference
pEGKG	<i>GAL1_{prom}</i> -GST 2 μ m URA3	162
pEGKG-Rod1	<i>GAL1_{prom}</i> -GST 2 μ m URA3	163
pEGKG-Rog3	<i>GAL1_{prom}</i> -GST 2 μ m URA3	163
pEGKG-Ldb19	<i>GAL1_{prom}</i> -GST 2 μ m URA3	163
pEGKG-Art5	<i>GAL1_{prom}</i> -GST 2 μ m URA3	163
pEGKG-Rod1 ^{PANA} (pJT4954) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rod1 ^{PASA} (pJT4955) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rod1 ^{PPXY-less} (pJT4956) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rod1 ^{4KR} (Rod1 ^{K235R K245R K264R K267R}) (pJT5045) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rog3 ^{PANA} (pJT4958) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rog3 ^{PASA} (pJT4959) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rog3 ^{PPXY-less} (pJT4960) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rog3 ^{VASA} (pJT4978) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rog3 ^{V/PPXY-less} (pJT4979) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rog3 ^{Δ400} (pJT4983) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rog3 ^{4KR} (Rog3 ^{K235R K245R K264R K267R}) (pJT5060) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pEGKG-Rod1 ^{AQAKAA} (pJT4957) ^a	<i>GAL1_{prom}</i> -GST 2 μ m URA3	This study
pRS313	<i>CEN HIS3</i>	164
pRS313-Ldb19 (pJT4963) ^b	<i>LDB19_{prom}</i> <i>CEN HIS3</i>	This study
pRS313-Ldb19 ^{PALA} (pJT4964) ^c	<i>LDB19_{prom}</i> <i>CEN HIS3</i>	This study
pRS313-Ldb19 ^{PPCY} (pJT4965) ^c	<i>LDB19_{prom}</i> <i>CEN HIS3</i>	This study
pRS313-Ldb19 ^{PPXY-less} (pJT4966) ^c	<i>LDB19_{prom}</i> <i>CEN HIS3</i>	This study
pRS313-Ldb19 ^{K486R} (pJT5001) ^c	<i>LDB19_{prom}</i> <i>CEN HIS3</i>	This study
pGEX4T1-GST-Ste2 ²⁹⁷⁻⁴³¹	<i>T7 AMP</i>	163

^a Generated by site-directed mutagenesis (67) with synthetic oligonucleotides containing the desired codon alterations (using the wild-type sequence in pRS313 vectors as the template). DNA from the corresponding gene was amplified from genomic DNA by PCR (66) and then cloned into pEGKG.

^b DNA of the corresponding gene was amplified from genomic DNA by PCR (66) and then cloned into the XmaI-NotI sites in pRS313 (164).

^c Generated by site-directed mutagenesis with synthetic oligonucleotides containing the desired codon alterations and the corresponding *LDB19* DNA inserted into pRS313 as the template.

tration) for 10 min and then incubated for 1 h either with vehicle alone (90% [vol/vol] ethanol and 10% [vol/vol] aqueous Tween 20; designated ET) or with the potent and specific calcineurin inhibitor FK506 at a final concentration of 1 μ g/ml (added from a 10-mg/ml stock dissolved in ET) prior to galactose induction. After further incubation for 3.5 h, the cells were harvested and lysed by vigorous vortex mixing with glass beads in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol [DTT], 50 mM Tris-HCl [pH 7.4]) containing protease inhibitors (77). After brief centrifugation to remove unbroken cells and debris, the clarified extracts were incubated with glutathione-agarose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 2 h at 4°C and washed 3 times with 500 μ l RIPA buffer. Bound proteins were eluted from the beads in SDS-PAGE sample buffer and resolved by SDS-PAGE, and the presence of Cna1-GFP and GST-Rod1 was analyzed by immunoblotting.

To assess *in vivo* phosphorylation of Rod1, pEGKG-Rod1 and pEGKG-Rod1^{AQAKAA} were introduced into BY4741 and otherwise isogenic *cnb1 Δ* and *cna1 Δ cna2 Δ* derivatives by DNA-mediated transformation (65). Transformants were grown to mid-exponential phase in SC-2% raffinose. Cells were then treated with either ET alone or 1 μ g/ml FK506 in ET for 1 h, and then α -arrestin expression was induced by addition of 4% galactose (final concentration) followed by growth at 30°C for 4 h. Ten minutes prior to harvesting, the cultures were treated with 200 mM CaCl₂ (final concentration) to activate calcineurin. Protein extracts were prepared by glass bead lysis in RIPA buffer containing both 600 mM NaCl (HS-RIPA) and 1 μ g/ml FK506. After clarification by centrifugation, GST-Rod1 and GST-Rod1^{AQAKAA} were collected from the lysates by binding to glutathione-agarose for 2 h at 4°C. The beads were washed 2 times with 500 μ l HS-RIPA, and aliquots of the bead-bound proteins were incubated at 30°C for 45 min either in phosphatase buffer alone (1 mM MnCl₂, 10 mM NaCl, 2 mM DTT, 0.01% Brij-35, 50 mM HEPES [pH

7.5]) or in the same buffer containing 200 U of lambda phosphatase (New England BioLabs) in either the absence or presence of phosphatase inhibitors (10 mM Na₄P₂O₇, 10 mM NaF, 0.4 mM NaVO₃, 0.4 mM Na₃VO₄, and 0.1 mM glycerol-3-phosphate). Supernatant liquid was removed by aspiration. Bound protein was eluted at 37°C for 15 min in SDS-PAGE sample buffer, resolved by SDS-PAGE (6% acrylamide gel), and analyzed by immunoblotting.

Assessment of Rsp5 copurification with α -arrestins was performed as described previously (78). To assess the state of α -arrestin modification by ubiquitin *in vivo*, BJ5459 *GEV* cells carrying a plasmid vector (pEGKG) for the expression of GST-Ldb19 or GST-Ldb19(K486R) were grown to mid-exponential phase and induced with 20 μ M β -estradiol (final concentration) for 3 h. After harvesting by centrifugation, the cells were washed and frozen in liquid N₂. Cell pellets were resuspended in 600 μ l immunoprecipitation buffer (100 mM NaCl, 0.2% Triton X-100, 15 mM EGTA, 50 mM Tris [pH 7.4]) containing 5 mM *N*-ethylmaleimide (NEM) and protease inhibitors (1 tablet of cOmplete protease inhibitor cocktail [Roche Applied Science] per 15 ml) and lysed at 4°C by vigorous vortexing with \sim 1-g glass beads (0.5 mm; BioSpec Products). After clarification, GST-tagged proteins were recovered from equal volumes of these extracts by incubation with GST-agarose beads for 2 h at 4°C. After two washes with coimmunoprecipitation buffer containing 150 mM NaCl, liquid was removed by aspiration and the beads were resuspended in SDS-PAGE sample buffer to elute the bound proteins, which were resolved by SDS-PAGE and analyzed by immunoblotting.

In vitro ubiquitinylation. The ability of α -arrestins to serve as substrates for Rsp5-mediated ubiquitinylation *in vitro* was assessed by minor modifications of previous methods (79). Briefly, GST-Rsp5 and a catalytically inactive mutant (GST-Rsp5^{C777A}) were expressed in and purified from *Escherichia coli* and the GST tag removed by cleavage with commercial rhinovirus 3C protease (PreScission; GE Healthcare). Uba1 (E1) and

Ubc1 (E2) were purified from yeast as previously described (80). Plasmid DNA (1 μ g) of a vector (pME32) carrying the open reading frames for either *ROD1*, *ROG3*, *LDB19*, or cognate derivatives containing mutated versions of one or more of their P/VPXY motifs was used as the template to generate the corresponding [35 S]methionine ([35 S]Met)-labeled protein by coupled *in vitro* transcription-translation using the TNT quick coupled system (Promega, Sunnyvale, CA). The resulting translation mixture was treated with a final concentration of 10 mM NEM for 15 min at room temperature to inactivate the deubiquitinating enzymes and ubiquitin-conjugating enzymes in the rabbit reticulocyte lysate (81). After quenching unreacted NEM with a final concentration of 20 mM DTT, portions (42 μ l) of each *in vitro* translation product were added to a reaction mixture (60 μ l final volume) containing the following components at the indicated final concentrations: 600 μ M ubiquitin (Sigma, St. Louis, MO), 0.5 mM ATP, 220 nM Uba1, and 3 μ M Ubc1. An aliquot (10 μ l) was removed as the zero time point, and the reaction was initiated immediately thereafter by addition of GST-Rps5 or, as a control, GST-Rps5^{C777A} (100 nM final concentration). Additional aliquots were removed at 5, 15, and 30 min, and each was quenched by immediate mixing with 8 \times concentrated SDS-PAGE sample buffer followed by incubation at 37°C for 15 min. The resulting products were resolved by SDS-PAGE (4.5% acrylamide gel). After drying the gel, the radioactive species were detected by exposure to a phosphorimager screen for 2 h followed by visualization on a Typhoon FLA 7000 laser scanner (GE Healthcare).

Fluorescence microscopy. Imaging of Ste2-mCherry was performed as described previously (21). Imaging of Ste2-GFP was carried out as described previously (82), using an inverted fluorescence microscope (Axiovert 200; Carl Zeiss GmbH, Jena, Germany) equipped with a charge-coupled-device (CCD) camera (Sensicam; PCO-Tech, Inc., Romulus, MI), an X-Cite 120 PC fluorescence illumination system (Exfo Ltd., Quebec, Canada), and a 100 \times (1.4-numeric-aperture) Plan-Apochromat objective. The day before examining the cells, cultures of the strains to be tested were streaked at a low dilution on plates containing minimal medium (yeast nitrogen base), supplemented with appropriate nutrients to select for plasmid maintenance (if needed), and grown overnight at 30°C, yielding small colonies in mid-exponential-phase growth (as judged by the presence of cells in all cell cycle stages in the population). Single colonies were suspended in 2.75 μ l of the same medium on the surface of a glass slide under a coverslip immediately before imaging at room temperature using Slidebook software (v5.0.0.32; Olympus America, San Jose, CA) with identical imaging parameters (2 by 2 binning, 500-ms exposure) for all samples. Images were processed using ImageJ (v1.48b) with identical maximum and minimum intensity values applied to all images.

RESULTS

Ldb19, Rod1, and Rog3 negatively regulate the mating pathway.

We first tested whether any of 12 of the 14 recognized yeast α -arrestins affects the function of the Ste2 GPCR. To explore this possibility, we tested derivatives of a *MATa* strain (BY4741) harboring an α -arrestin deletion for their response to mating pheromone using a standard agar diffusion bioassay. We did not examine Spo23 (83), which is expressed only in meiotic cells (84), or Bul3, which can be expressed only by translational readthrough of a stop codon situated between two adjacent open reading frames (85). We also tested strains containing deletions of both members of paralogous pairs of α -arrestins or deletions of 9 α -arrestins in combination (*9arr Δ*) (58). A modest but readily detectable and reproducible increase in pheromone sensitivity (as judged by the diameter of the halo of G_1 -arrested cells) was observed for the *9arr Δ* mutant, an *ldb19 Δ* mutant, and a *rod1 Δ rog3 Δ* double mutant (Fig. 1A and B) (however, not *rod1 Δ* or *rog3 Δ* single mutants [data not shown]). Rod1 and Rog3 share greater similarity to each other (45% identity) than to any other α -arrestin (\leq 30% identity), suggesting that this pair has an overlapping function. None

of the other single deletions or deletions of any of the other four paralogous pairs, e.g., *aly1 Δ aly2 Δ* (Aly1/Art6 and Aly2/Art3 share 42% identity), exhibited a change in halo size (Fig. 1A, upper, and B). Thus, the effects observed were specific to just three α -arrestins, Ldb19, Rod1, and Rog3.

Neither the *ldb19 Δ* mutant nor the *rod1 Δ rog3 Δ* double mutant exhibited the same increase in halo size displayed by the *9arr Δ* cells; however, *ldb19 Δ* , *rod1 Δ* , and *rog3 Δ* are among the deletions carried by the *9arr Δ* cells. Hence, we constructed the corresponding triple mutant, and as anticipated, we found that the pheromone sensitivity of *ldb19 Δ rod1 Δ rog3 Δ* cells phenocopied that of the *9arr Δ* cells (Fig. 1A, lower, and B). These data suggest that the absence of these three α -arrestins is responsible for the observed behavior of the *9arr Δ* cells. Moreover, despite the fact that these cells possess all of the previously characterized mechanisms for recovery and adaptation, these three α -arrestins clearly contribute to downregulation of pheromone signaling. Furthermore, the fact that the effects of an *ldb19 Δ* mutation are additive to those of *rod1 Δ rog3 Δ* provides evidence that Ldb19 acts independently of Rod1 and Rog3.

Ldb19, Rod1, and Rog3 function independently from Bar1 and Sst2. Some α -arrestins have been implicated in aspects of vesicle-mediated transport other than endocytosis (86). Therefore, it was possible that lack of Ldb19, Rod1, and/or Rog3 enhances pheromone response simply by impeding the action of known negative regulators of pheromone signaling that require membrane trafficking (such as the secreted α -factor protease Bar1/Sst1) or membrane interaction (such as the receptor- and G-protein-associated RGS protein Sst2). However, compared to an otherwise isogenic *bar1 Δ* mutant, an *ldb19 Δ bar1 Δ* double mutant, a *rod1 Δ rog3 Δ bar1 Δ* triple mutant, and an *ldb19 Δ rod1 Δ rog3 Δ bar1 Δ* quadruple mutant exhibited significantly greater pheromone sensitivity at every concentration of α -factor tested (Fig. 1C). Because Bar1 is absent from these cells, the observed additive behavior shows that the increase in pheromone sensitivity has nothing to do with preventing efficient Bar1 secretion or function. Likewise, compared to an otherwise isogenic *sst2 Δ* mutant, an *ldb19 Δ sst2 Δ* double mutant, a *rod1 Δ rog3 Δ sst2 Δ* triple mutant, and an *ldb19 Δ rod1 Δ rog3 Δ sst2 Δ* quadruple mutant exhibited greater pheromone sensitivity at every concentration of α -factor tested (Fig. 1D). Again, this additive effect indicates that the absence of Ldb19, Rod1, and/or Rog3 does not cause increased pheromone sensitivity by interfering with Sst2 action. Because these α -arrestins negatively regulate pheromone response by a mechanism(s) independent from those exerted by either Bar1 or Sst2, we used *bar1 Δ* and *sst2 Δ* cells as sensitized backgrounds in which to further characterize the function of Ldb19, Rod1, and Rog3.

Rod1 and Rog3 promote adaptation. The increased pheromone sensitivity observed when Ldb19 or Rod1 and Rog3 are absent is consistent with a role for these proteins in receptor downregulation and/or signal dampening. If so, overexpression of such negative regulators should stimulate recovery from pheromone signaling. The agar diffusion bioassay provides a convenient means to assess desensitization, because adaptation is readily monitored by examining the rate and extent of the resumption of cell growth inside the initial zone of pheromone-imposed G_1 arrest. Normally, in the absence of Sst2, once cells are exposed to pheromone, little or no adaptation is observed even after prolonged incubation (19, 21); only occasional papillae arise (see, for

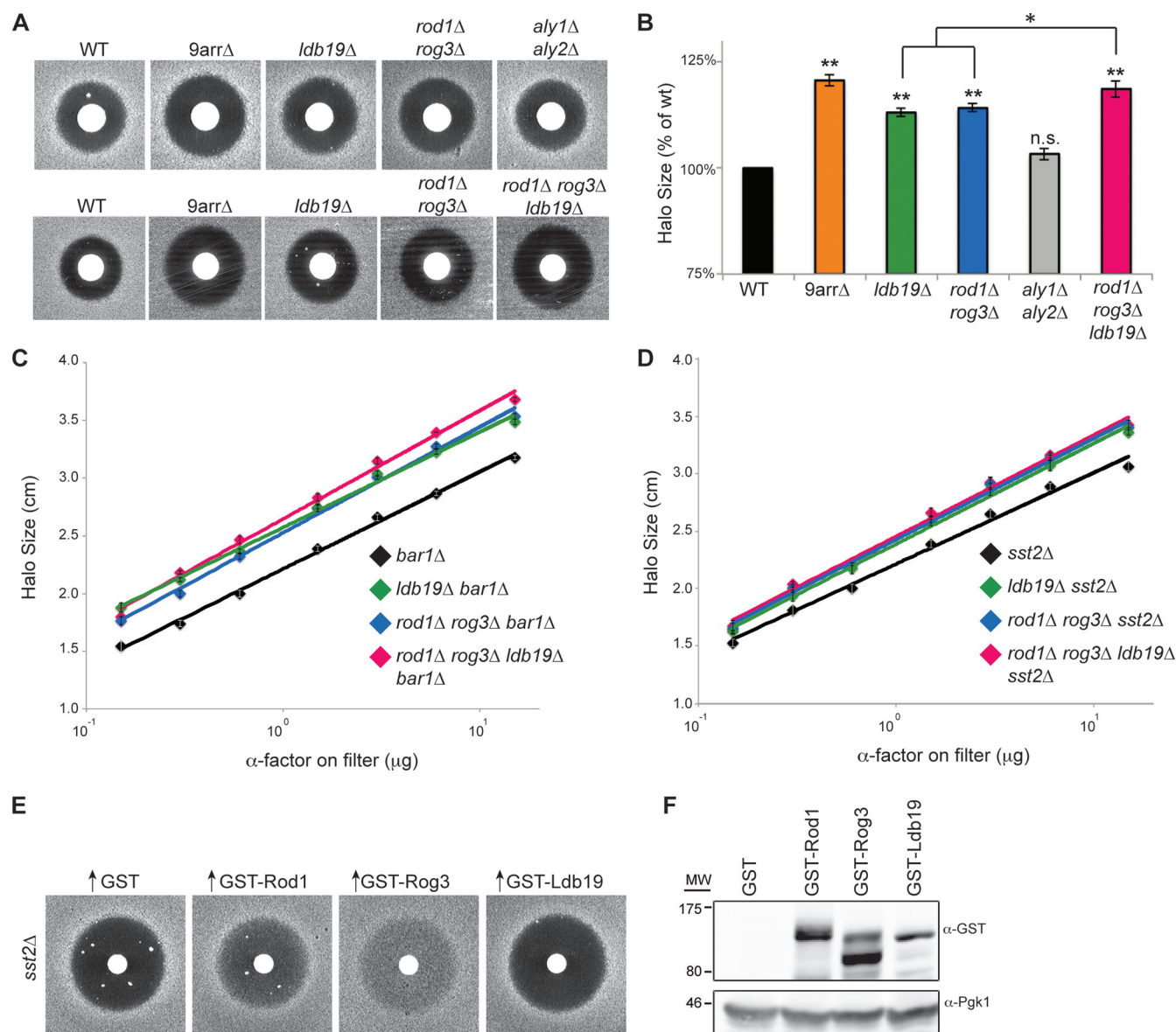


FIG 1 Specific α -arrestins negatively regulate pheromone signaling and act independently from secreted protease Bar1 and RGS protein Sst2. (A) Pheromone sensitivity of wild-type *MATa* cells (BY4741) and otherwise isogenic derivatives containing the indicated α -arrestin deletions (*9arrΔ*, EN60; *ldb19Δ*, BY4741 *ldb19Δ*; *rod1Δ rog3Δ*, JT5858; *aly1Δ aly2Δ*, D2-6A; *rod1Δ rog3Δ ldb19Δ*, JT6675) was assessed by the agar diffusion (halo) bioassay for α -factor-induced growth arrest on YPD medium (15 μ g α -factor spotted on each filter disk). Data from one representative experiment are shown. (B) Quantification and statistical analysis of the change in halo diameter, determined as described for panel A, for independent replicate experiments ($n = 4$). The average halo diameter for control cells was set at 100%, and halo sizes for each mutant were normalized to the control. Error bars indicate \pm standard errors of the means (SEM); **, $P < 0.0001$; *, $P < 0.05$; n.s., value not statistically significant. (C) Pheromone sensitivity of a *MATa bar1Δ* strain (JT5915) and otherwise isogenic *ldb19Δ bar1Δ* (JT5916), *rod1Δ rog3Δ bar1Δ* (JT5917), and *rod1Δ rog3Δ ldb19Δ bar1Δ* (JT6674) derivatives was determined as described for panel A in response to the indicated amounts of α -factor (150 ng to 15 μ g). Values represent the averages from independent replicate experiments ($n = 5$); errors bars indicate \pm SEM. (D) Pheromone sensitivity of a *MATa sst2Δ* strain (JT6755) and otherwise isogenic *ldb19Δ sst2Δ* (JT6660), *rod1Δ rog3Δ sst2Δ* (JT6702), and *rod1Δ rog3Δ ldb19Δ sst2Δ* (JT6662) derivatives was determined in response to the indicated amounts of α -factor. Values represent the averages from independent replicate experiments ($n = 3$); errors bars indicate \pm SEM. (E) Pheromone sensitivity of a *MATa sst2Δ* strain (JT5919) carrying the GEV chimera for β -estradiol-induced expression of genes under *GAL* promoter control and containing either empty vector (high-copy-number *URA3*-marked 2 μ m DNA plasmid) or the same vector harboring the indicated α -arrestin (as a fusion to GST) under *GAL* promoter control was determined on SC-Ura, as described for panel A, using 15 μ g of α -factor spotted on the filter disk after induction with β -estradiol (see Materials and Methods). Data from one representative experiment ($n = 5$) are shown. (F) Confirmation of α -arrestin expression. Proteins from whole-cell extracts of the cells shown in panel E were prepared, resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Data from one representative experiment ($n = 5$) are shown. MW, molecular weight in thousands.

example, Fig. 1E, left), which represent rare cells with a selective advantage (they became pheromone resistant by acquiring a spontaneous *ste* mutation) (87). Remarkably, overexpression of either GST-Rod1 or GST-Rog3 in *sst2Δ* cells caused turbid halos diag-

nostic of adaptation, recovery from G₁ arrest, and a return to cell growth (Fig. 1E). Similar turbid halos have been observed when other negative regulators of pheromone response are overexpressed (88). Although both α -arrestins are produced at an equiv-

alent level (Fig. 1F), the adaptation-promoting effect of GST-Rog3 was reproducibly more potent than that of GST-Rod1.

In contrast, overexpression of GST-Ldb19, or untagged Ldb19 (data not shown), in *sst2Δ* cells did not promote adaptation (Fig. 1E). In multiple trials, GST-Ldb19 expression was always lower than that of GST-Rod1 and GST-Rog3 (Fig. 1F). Therefore, it was formally possible that the level of GST-Ldb19 achieved was insufficient to support adaptation. However, like GST-Ldb19, other GST-tagged α -arrestins that are expressed at a level comparable to or even higher than that of GST-Rod1 and GST-Rog3 (e.g., GST-Art5 and GST-Csr2/Art8) also failed to promote adaptation in *sst2Δ* cells (data not shown). These observations argue, first, that the adaptation-promoting effects of Rod1 and Rog3 are specific. Second, and tellingly, because α -factor is present continuously in these assays, these observations indicate that Rod1 and Rog3 act on the pheromone receptor in its ligand-occupied conformation, whereas Ldb19 is unable to do so. These data also show that Rod1 and Rog3 act at a different level and/or via a different mechanism than Ldb19. Of course, some other factor may be rate limiting for the Ldb19-promoted adaptation pathway (such as a stimulus that results in more receptor misfolding or unfolding [see Discussion]).

Absence of α -arrestins and mating pathway signaling. Pheromone-imposed G_1 arrest is one measure of mating pathway function. We used two other independent methods to confirm that absence of α -arrestins leads to an enhanced pheromone response. First, under conditions where the concentration of α -factor remains essentially constant (cells carried a *bar1Δ* mutation), we compared the kinetics of activation (via dual phosphorylation) of Fus3 (89), the mating pheromone response pathway-specific MAPK (12, 90), after exposing an otherwise wild-type strain and an *ldb19Δ rod1Δ rog3Δ* triple mutant to α -factor (Fig. 2A). As observed in the halo bioassay, there was more efficacious and sustained signaling in cells lacking the three α -arrestins than in the control cells, although the effect was relatively modest. *FUS3* itself is known to be a pheromone-induced gene product (27, 89, 91), and the level of Fus3 increased in control cells and cells lacking Ldb19, Rod1, and Rog3. However, the fraction of Fus3 in its activated state was higher and more persistent in the cells lacking the three α -arrestins than in the control cells (Fig. 2B).

Another standard used to measure pheromone response is induction of the pheromone-responsive gene *FUS1* (92). We quantified the expression level of an integrated *FUS1_{prom}*-eGFP reporter gene (73) in *bar1Δ* cells that were otherwise wild type or carried the *ldb19Δ rod1Δ rog3Δ* mutations using flow cytometry. It was shown previously that basal signaling in the mating pheromone response pathway arises largely from stochastic spontaneous dissociation of receptor-heterotrimeric G protein complexes (93, 94). An increase in receptor level in the PM shifts the equilibrium toward complex formation and reduces basal signaling (21, 23). For this reason, if the α -arrestin-deficient cells internalize Ste2 less efficiently, then basal signaling should be reduced. Consistent with this prediction, basal expression was significantly lower in cells lacking Ldb19, Rod1, and Rog3 than in control cells (Fig. 2C). Nonetheless, after exposure to α -factor, the level of *FUS1* expression achieved in the α -arrestin-deficient cells was nearly equivalent to that in the wild type. Therefore, the induction ratio for the *FUS1* reporter was ~ 3 -fold higher in the *ldb19Δ rod1Δ rog3Δ bar1Δ* cells than in *bar1Δ* cells (Fig. 2D). Thus, as judged by three different assays, pheromone signaling is more

sustained in cells lacking Ldb19, Rod1, and Rog3 than in control cells, consistent with loss of negative regulation of the pathway.

Efficient Ste2 internalization requires Ldb19, Rod1, and Rog3. In *S. cerevisiae*, various classes of integral polytopic PM proteins can be endocytosed by clathrin-dependent (95) and clathrin-independent (96) routes. Caveolin-like structures in yeast (“eisosomes”) (97, 98) do not appear to be sites of endocytosis (99, 100) and may even protect cargo from internalization (101). The evidence that various yeast α -arrestins are necessary for efficient internalization of distinct PM-localized nutrient permeases is compelling (57–59, 102). If Ldb19, Rod1, and/or Rog3 contributes to GPCR downregulation by any endocytic route, one would expect to detect some Ste2 accumulation at the PM in cells lacking one or more of these α -arrestins. To examine receptor localization, we first used full-length Ste2 tagged at its C terminus with mCherry (21). We showed before that in otherwise wild-type cells (even in the absence of pheromone), the red fluorescence resides mainly in the vacuole (21), presumably because maturation of the mCherry chromophore is slow relative to the rate of constitutive endocytosis and/or because the mCherry portion of the fusion persists due to its slow degradation. Indeed, in wild-type cells, the fluorescent signal was confined almost exclusively to the vacuole (Fig. 3A, left), whereas in isogenic *ldb19Δ* single, *rod1Δ rog3Δ* double, and *ldb19Δ rod1Δ rog3Δ* triple mutants, fluorescence was discernible at the PM (Fig. 3A).

To confirm that these conclusions were not dependent on the chromophore used to monitor Ste2 localization and to quantify the results, the same analysis was carried out using cells expressing full-length Ste2 tagged at its C terminus with eGFP. Again, the fluorescent signal in the parental strain was confined largely to the vacuole in virtually every cell, whereas the vast majority of the *9arrΔ*, *ldb19Δ*, *rod1Δ rog3Δ*, and especially *ldb19Δ rod1Δ rog3Δ* cells exhibited readily detectable PM fluorescence (Fig. 3B). In marked contrast, the level of Ste2–GFP fluorescence at the PM was unchanged in mutants lacking other α -arrestins, e.g., *aly1Δ aly2Δ* (Fig. 3B). These visual impressions were corroborated by averaging the intensity of PM fluorescence determined by taking multiple line scans across large numbers of cells in each field (Fig. 3C). Together, these results are consistent with Ldb19, Rod1, and Rog3 acting as negative regulators of pheromone-initiated signaling by promoting efficient Ste2 internalization.

The ubiquitin ligase Rsp5 is required for Ldb19 and Rod1 action. Aside from an N-terminal arrestin fold that mediates interaction with specific targets, a defining hallmark of an α -arrestin is that its C-terminal sequence contains multiple copies of PPXY (and/or variants thereof, such as LPXY and VPXY) (Fig. 4A). The PPXY motifs serve as docking sites for binding three tandem WW domains (103) present in the HECT family ubiquitin ligase (E3) Rsp5 (104, 105). In this way, α -arrestins act as adaptors that link PM substrates that lack endogenous PPXY motifs to Rsp5 (106, 107), which catalyzes substrate ubiquitylation, thereby marking cargo for endocytosis (57, 58, 102, 108). The primary structure of Ste2 has no PPXY motif or variant thereof in its cytoplasmic loops or cytosolic tail, yet it becomes ubiquitylated in an Rsp5-dependent manner on seven Lys residues in its C-terminal tail (21, 41, 55).

To determine whether Rsp5 recruitment is required for negative regulation of pheromone signaling by Ldb19, Rod1, or Rog3, we first mutated the two PPXY motifs in each of these α -arrestins to PAXA and then used two different methods to assess whether

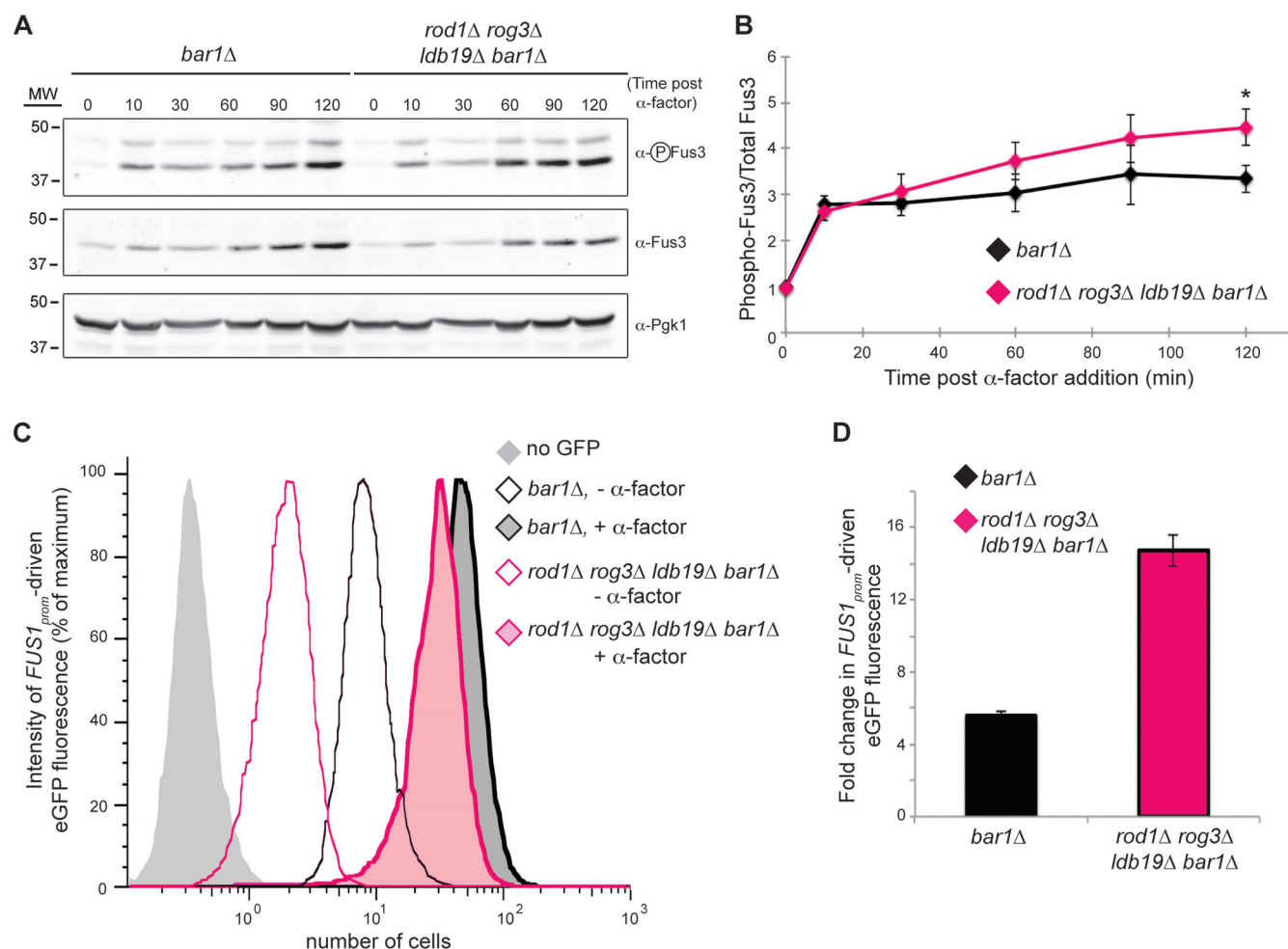


FIG 2 Pheromone signaling is more persistent in cells lacking Ldb19, Rod1, and Rog3. (A) Cultures of a *MATa bar1Δ* strain (JT5915) and an otherwise isogenic *ldb19Δ rod1Δ rog3Δ bar1Δ* derivative (JT6674) were grown to mid-exponential phase and then treated with 15 nM α -factor for the indicated times. Samples were withdrawn at the indicated time points and rapidly chilled on ice. The cells were collected by centrifugation and extracted, and proteins in the resulting whole-cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. Data from one representative experiment ($n = 3$) are shown. MW, molecular weight in thousands. (B) Values represent the mean pixel intensities for the phosphorylated and total Fus3 bands, determined as described for panel A, from the three independent replicate experiments. Error bars indicate \pm SEM; *, $P < 0.01$. (C) Cultures of a *MATa bar1Δ FUS1_{prom}-eGFP* strain (JT6686) and an otherwise isogenic *ldb19Δ rod1Δ rog3Δ bar1Δ FUS1_{prom}-eGFP* derivative (JT6668) were grown to mid-exponential phase. Samples of these cultures were withdrawn, and the distribution of fluorescent cells was determined using a fluorescence-activated cell sorter (model FC500; Beckman-Coulter) at the Flow Cytometry Facility of the UC Berkeley Cancer Research Laboratory. The remainder of each culture was treated with 15 nM α -factor for 2 h, and the profile of fluorescent cells in each culture was redetermined. (D) The average fold change in the level of GFP fluorescence determined from the ratio of the areas under the curves of uninduced and pheromone-induced cells of the indicated genotypes for independent replicate experiments ($n = 3$) performed as described for panel C. Error bars indicate \pm SEM.

these point mutations successfully abrogated interaction with Rsp5. In the case of Rog3, we also mutated its VPXY motif because an identical motif in α -arrestin Rim8/Art9 was shown to mediate Rsp5 association (109). As observed for other α -arrestins (57, 78, 108), we found that Rsp5 efficiently copurified with GST-Ldb19, GST-Rod1, and GST-Rog3 (and not with a GST control), whereas the corresponding PPXY-less (or, in the case of Rog3, P/VPXY-less) mutants exhibited dramatic decreases in the amount of Rsp5 recovered: Ldb19, 93% reduction; Rod1, 71% reduction; and Rog3, 83% reduction (Fig. 4B). The residual amount of Rsp5 observed for the mutants does not represent residual interaction but rather nonspecific background, because no further reduction below this threshold was observed when a complete C-terminal truncation [Rog3(Δ 400-733)] was examined (Fig. 4B). As an independent and more sensitive indicator of the ablation of Rsp5

interaction by these point mutations, we took advantage of the fact that this E3 efficiently ubiquitinylates endogenous Lys residues in the α -arrestins (57, 58, 102, 108). We found that each of the three α -arrestins, prepared by coupled *in vitro* transcription and translation, served as an efficient substrate for Rsp5-dependent ubiquitinylation, as judged by complete conversion of the starting material to a broad spectrum of polyubiquitinated, slower-mobility species (Fig. 4C). In control reactions incubated with Rsp5^{C777A}, no detectable ubiquitinated species were observed (data not shown). As expected, the PPXY point mutations abolished Rsp5-dependent modification of each α -arrestin almost entirely (Fig. 4C). Thus, as judged by two different criteria, the point mutants we generated in Ldb19, Rod1, and Rog3 clearly compromise their interaction with Rsp5.

We then used a complementation test to determine if Rsp5

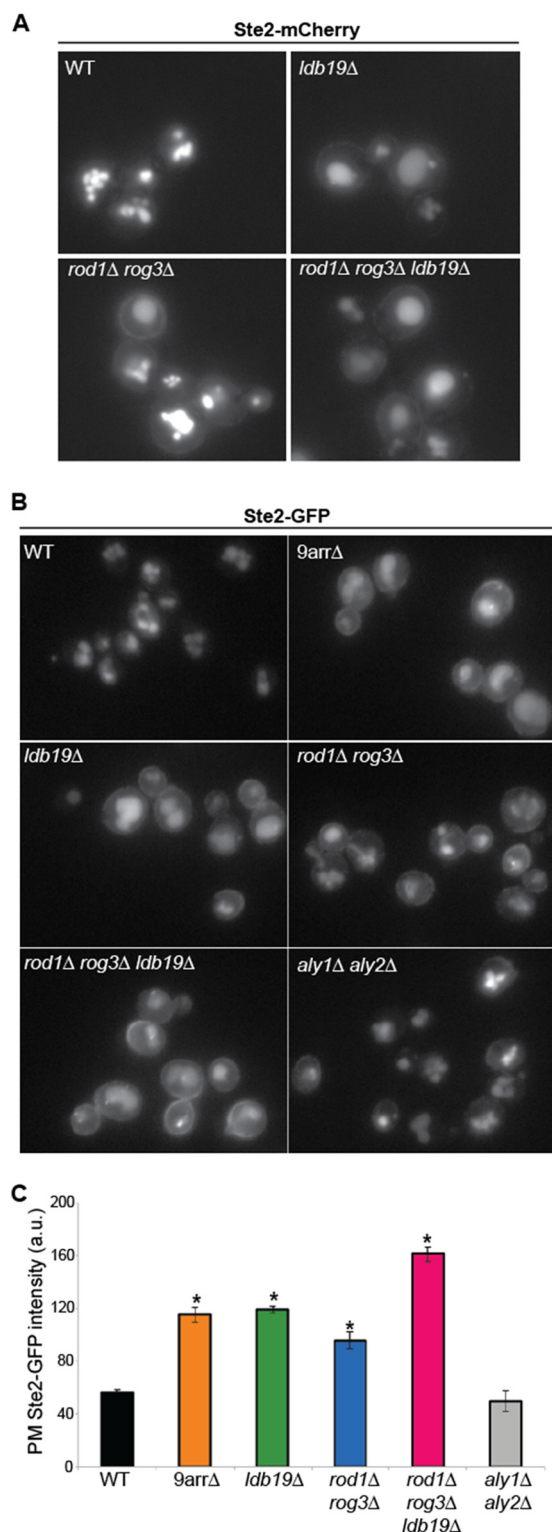


FIG 3 Increased abundance of Ste2 in the plasma membrane in cells lacking Ldb19 and/or Rod1 and Rog3. (A) A *MATa* strain expressing an integrated copy of Ste2-mCherry (as the sole copy of this receptor) from the native *STE2* promoter at the endogenous *STE2* locus on chromosome VI (JT6677) and otherwise isogenic *ldb19*Δ (JT6678), *rod1*Δ *rog3*Δ (JT6679), and *rod1*Δ *rog3*Δ *ldb19*Δ (JT6680) derivatives were examined by fluorescence microscopy. Representative images were recorded as described in Materials and Methods. (B) The same as panel A, except the cells expressed an integrated copy of Ste2-GFP

association is required for Ldb19 function in pheromone signal dampening. As described earlier (Fig. 1A), *ldb19*Δ *bar1*Δ cells display a halo of pheromone-induced growth inhibition that is larger than that of isogenic *bar1*Δ cells (Fig. 5A). Reintroduction of wild-type *LDB19* (expressed from its native promoter on a low-copy-number *CEN* plasmid) into the *ldb19*Δ *bar1*Δ strain reduced the halo size to that observed in the *bar1*Δ strain, whereas even single PAXA point mutations and the double (PPXY-less) mutant were unable to do so (Fig. 5A and B). Thus, the ability of Ldb19 to down-modulate signaling requires its interaction with Rsp5.

To determine if Rsp5 association is required for either Rod1 or Rog3 function in signal dampening, we exploited their ability to promote recovery and adaptation in *sst2*Δ cells exposed to pheromone, as described earlier (Fig. 1E). Overexpression of wild-type *ROD1* (as a GST fusion from the *GAL1* promoter on a high-copy-number 2μm DNA plasmid) in *sst2*Δ cells produced a turbid halo and reduced halo size, whereas even single PAXA point mutations and the double (PPXY-less) mutant were unable to do so (Fig. 5C, top), even though the Rod1 mutants were expressed at a level as high as or higher than that of the wild-type protein (Fig. 5D). Thus, as for Ldb19, the ability of Rod1 to downregulate signaling also requires its interaction with Rsp5.

Strikingly, as judged by the same assay, overexpression in *sst2*Δ cells of wild-type Rog3, single PAXA mutants, and the double PPXY-less mutant (Fig. 5C, lower), and even the P/VPXY-less derivative lacking all three of its Rsp5-binding motifs (Fig. 5E) produced turbid halos, even though the P/VPXY mutations eliminated the ability of Rog3 to associate with (Fig. 4B) and be modified (Fig. 4C) by Rsp5. Hence, in contrast to Ldb19 and Rod1, the ability of Rog3 to squelch pheromone signaling does not obligatorily require association with Rsp5. However, it was possible that Rog3 associates with its closest paralog, Rod1, to form a heterodimer (or higher oligomer), and Rsp5 recruited by this partner overcomes the loss of the P/VPXY motifs in Rog3. However, this possibility was eliminated (as well as effects of Rog3 mediated through Ldb19) because overexpressed Rog3 and its PPXY-less derivative still promoted efficient adaptation in cells lacking endogenous Ldb19, Rod1, and Rog3 (Fig. 5E). Thus, negative regulation of pheromone signaling by Rog3 does not require interaction, either direct or indirect, with Rsp5. Indeed, just the arrestin fold domain at the N terminus of Rog3 is sufficient to promote desensitization, because overexpression of a truncation mutant, Rog3(Δ400-733), yielded halos just as turbid, if not more so, than those of full-length Rog3. In contrast, Ldb19(Δ447-818) and Rod1(402-837) were nonfunctional (data not shown). Therefore, Rog3 can act on the receptor to negatively regulate signaling by a mechanism that is independent of the other two α-arrestins and Rsp5.

Ubiquitylation of Ldb19, Rod1, and Rog3 is dispensable for signal dampening. It has been demonstrated that the cognate α-arrestin itself becomes ubiquitinated during the process of recruiting Rsp5 to nutrient permeases (57, 58, 78, 102, 108). Moreover, the Lys residues in Ldb19 and Rod1 that are ubiquiti-

as the sole source of the receptor, and *9arr*Δ (JT6757) and *aly1*Δ *aly2*Δ (JT6762) derivatives were also visualized. (C) Mean intensity of PM fluorescence was quantified for each of the indicated strains (≥50 cells each) using ImageJ and plotted in arbitrary units (a.u.). Values significantly different from those of the control cells were assessed using a one-way analysis of variance test with Tukey's *post hoc* comparison (165). *, *P* < 0.0001.

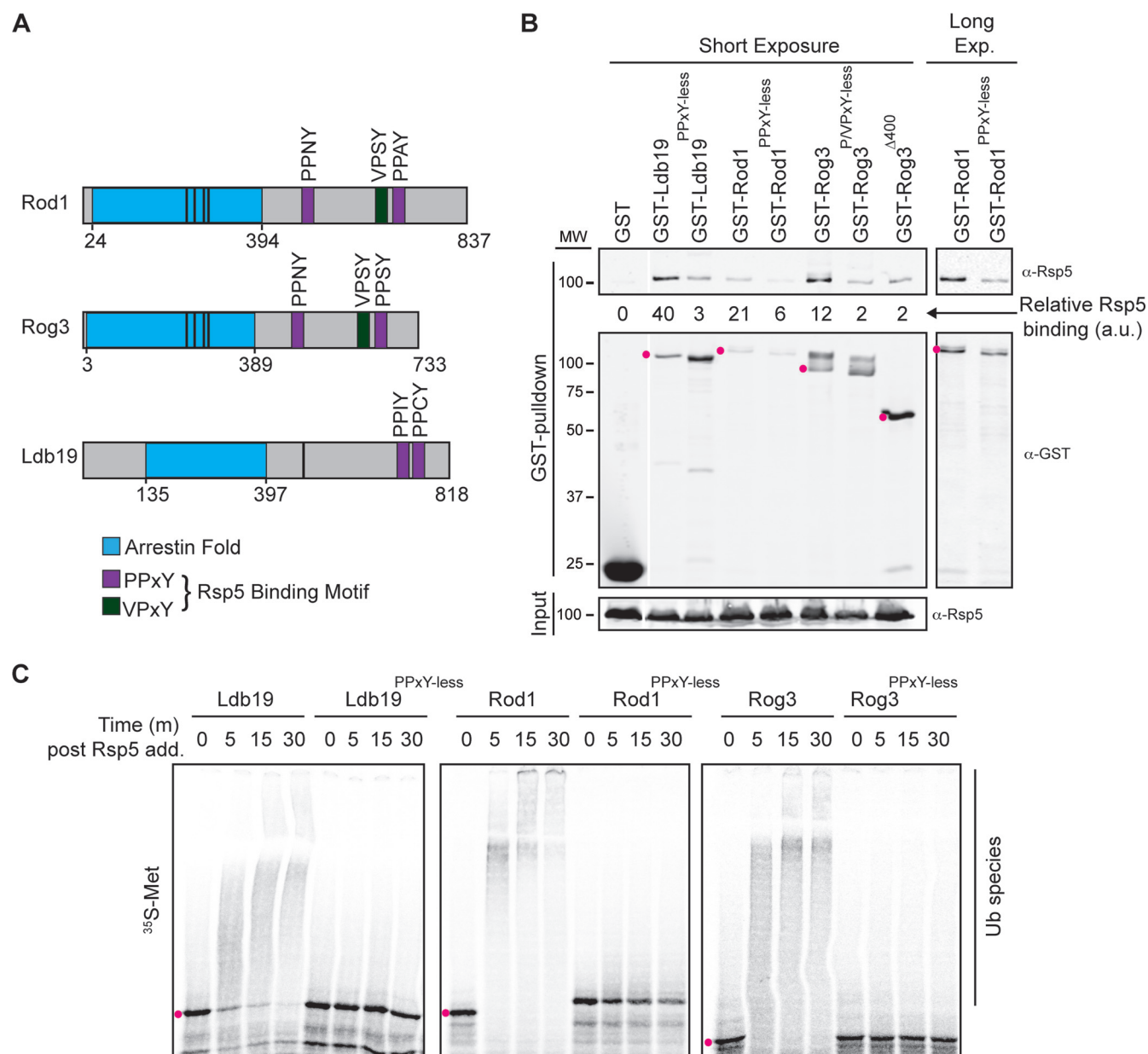


FIG 4 P/VPXY motifs in Ldb19, Rod1, and Rog3 are required for Rsp5 binding and Rsp5-mediated ubiquitinylation. (A) Schematic depiction of the primary structures of Ldb19, Rod1, and Rog3. Residues (numbers below each bar) comprising the arrestin fold (blue) in Ldb19 according to reference 57 and in Rod1 and Rog3 as predicted by the Phyre2 modeling algorithm (166) are shown, and positions of the consensus Rsp5-binding motifs, PPXY and VPXY, and reported ubiquitinylation sites (black lines) are indicated. (B) Cultures of a GEV derivative of vacuolar protease-deficient strain BJ5459 (160) expressing the indicated α -arrestin or the derived P/VPXY substitution mutant (as a GST fusion from the *GAL* promoter) were grown to mid-exponential phase. Protein expression was induced with β -estradiol, and the cells were harvested by centrifugation and ruptured by vigorous vortex mixing with glass beads. GST fusions in the resulting extracts (pink dots) were captured by binding to glutathione-agarose beads. After washing, the bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. MW, molecular weight in thousands; Long Exp., long exposure. (C) The Rsp5-catalyzed and time-dependent ubiquitinylation of an [^{35}S]Met-labeled α -arrestin or its cognate PPXY substitution mutant (pink dots), prepared by coupled *in vitro* transcription-translation, was performed and analyzed using a phosphorimager as described in Materials and Methods. add., addition; Ub, ubiquitinylation.

nylated have been mapped (Fig. 4A), and it was reported that corresponding K-to-R mutations in Ldb19 and Rod1 block endocytosis of their target nutrient permeases, the arginine transporter Can1 (57) and the lactate transporter Jen1 (102), respectively, suggesting that ubiquitinylation is required for the endocytosis-promoting function of these and other α -arrestins (108).

Our PPXY mutants of Ldb19, Rod1, and Rog3 should prevent

ubiquitinylation of both the α -arrestin and its cargo, because they cannot bind Rsp5. Hence, the failure of PPXY-less versions of Ldb19 (Fig. 5A) and Rod1 (Fig. 5C) to promote signal dampening could be due to defective ubiquitinylation rather than an inability to deliver Rsp5 to the receptor (and/or other targets). To distinguish between these possibilities, we generated K-to-R mutations in the ubiquitinylation sites in Ldb19 (56) and Rod1 (64) to selec-

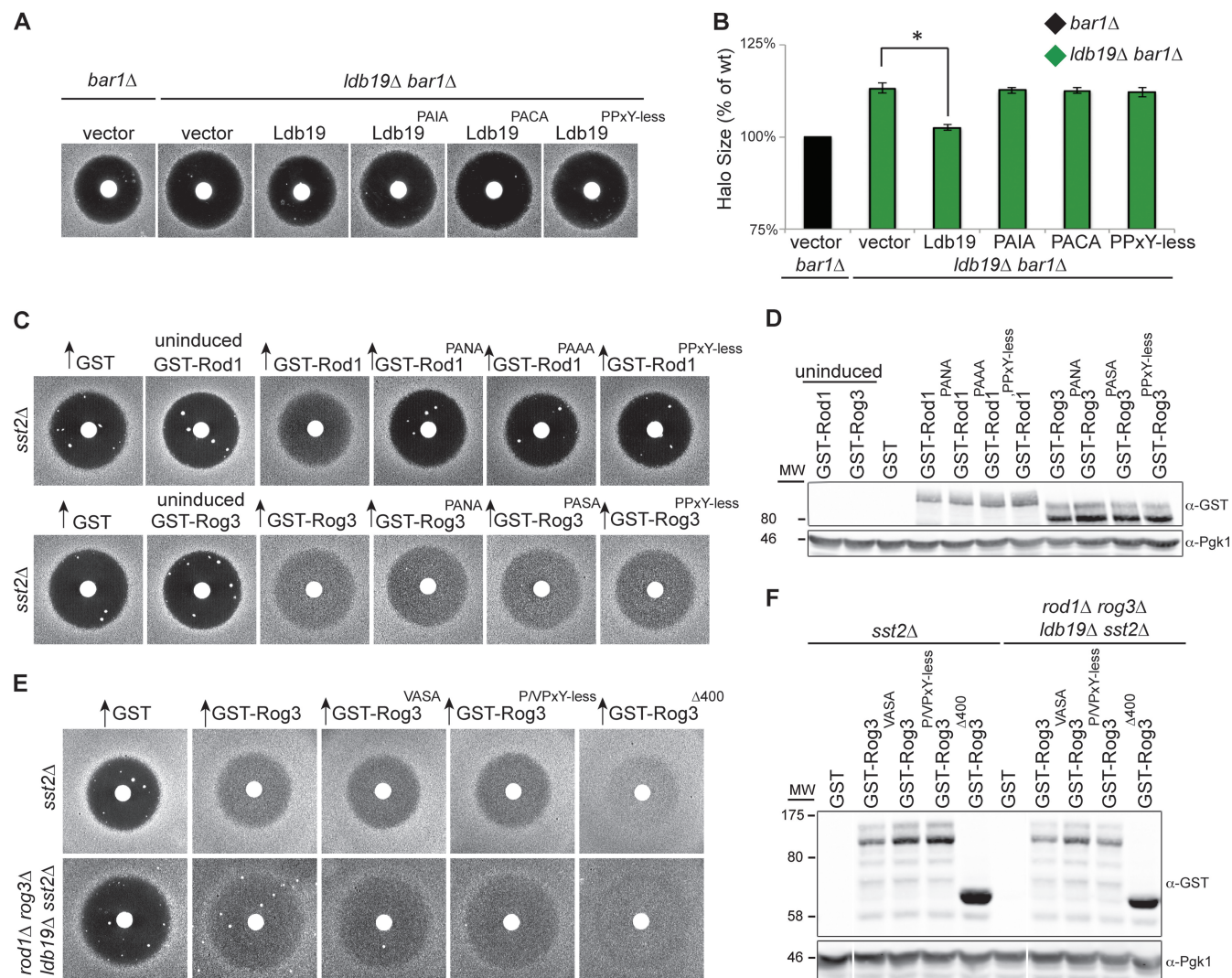


FIG 5 Ldb19 and Rod1, but not Rog3, require Rsp5 binding to downregulate pheromone signaling. (A) Pheromone sensitivity of a *MATa bar1Δ* strain (JT5915) and an otherwise isogenic *ldb19Δ bar1Δ* derivative (JT5916) carrying either empty vector (*HIS3*-marked *CEN* plasmid) or the same vector expressing wild-type *LDB19* or derivatives containing point mutations in each or both of its PPXY motifs binding was determined as described for Fig. 1A, except that the medium was SC-His. (B) Quantification and statistical analysis of the change in halo diameter, determined as described for panel A, for independent replicate experiments ($n = 3$). The average halo diameter for control cells was set at 100%, and the halo sizes for each mutant were normalized to that of the control. Error bars indicate \pm SEM; *, $P < 0.0001$. (C) Pheromone sensitivity of cultures of *MATa sst2Δ* GEV cells (JT5919) overexpressing either Rod1 or Rog3, as indicated, or the derived PPXY point mutants under the control of the *GAL* promoter on a high-copy-number *URA3*-marked 2 μ m DNA plasmid was determined as described in the legend to Fig. 1E. Data from one representative experiment ($n = 5$) are shown. (D) Proteins from whole-cell extracts of the cells shown in panel C were prepared, resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Data from one representative experiment ($n = 5$) are shown. MW, molecular weight in thousands. (E) Pheromone sensitivity of cultures of *MATa sst2Δ* GEV cells (JT5919) or an *ldb19Δ rod1Δ rog3Δ sst2Δ* GEV derivative (JT6716) overexpressing either Rog3 or the derived cognate P/VPXY point mutants, under the control of the *GAL* promoter on a high-copy-number *URA3*-marked 2 μ m DNA plasmid, was determined as described for panel C. Data from one representative experiment ($n = 3$) are shown. (F) Confirmation of protein expression, as described for panel D. Data from one representative experiment ($n = 3$) are shown.

tively disrupt their ubiquitinylation but leave Rsp5 binding intact. First, we generated GST-Ldb19(K486R) (57) and confirmed by pulldown, SDS-PAGE analysis, and immunoblotting that GST-Ldb19 is ubiquitinated *in vivo*, whereas GST-Ldb19(K486R) is not (Fig. 6A, upper). Strikingly, as judged by the complementation test, reintroduction of either wild-type *LDB19* or *LDB19*(K486R) into the *ldb19Δ bar1Δ* strain reduced the halo size to that observed in the *bar1Δ* strain (Fig. 6B and C). Thus, the ability of Ldb19 to down-modulate signaling requires Rsp5 interaction (Fig. 5A) but does not require its own ubiquitinylation.

We next generated GST-Rod1(K235R K245R K264R K267R),

here termed Rod1^{4KR}, based on published data about the ubiquitinylation sites in Rod1 (102), and confirmed that the 4K-to-R mutations abrogate its ubiquitinylation *in vivo* (Fig. 6A, lower). As assessed by the adaptation assay, overexpression of either wild-type *ROD1* or *ROD1*^{4KR} in *sst2Δ* cells produced equally turbid halos (Fig. 6D). Hence, the ability of Rod1 to downregulate signaling also requires Rsp5 interaction (Fig. 5C) but apparently not its own ubiquitinylation.

The Lys residues we mutated in Rod1 are conserved at the equivalent positions in Rog3; hence, we generated GST-Rog3(K235R K245R K264R K267R), here termed Rog3^{4KR}. In the

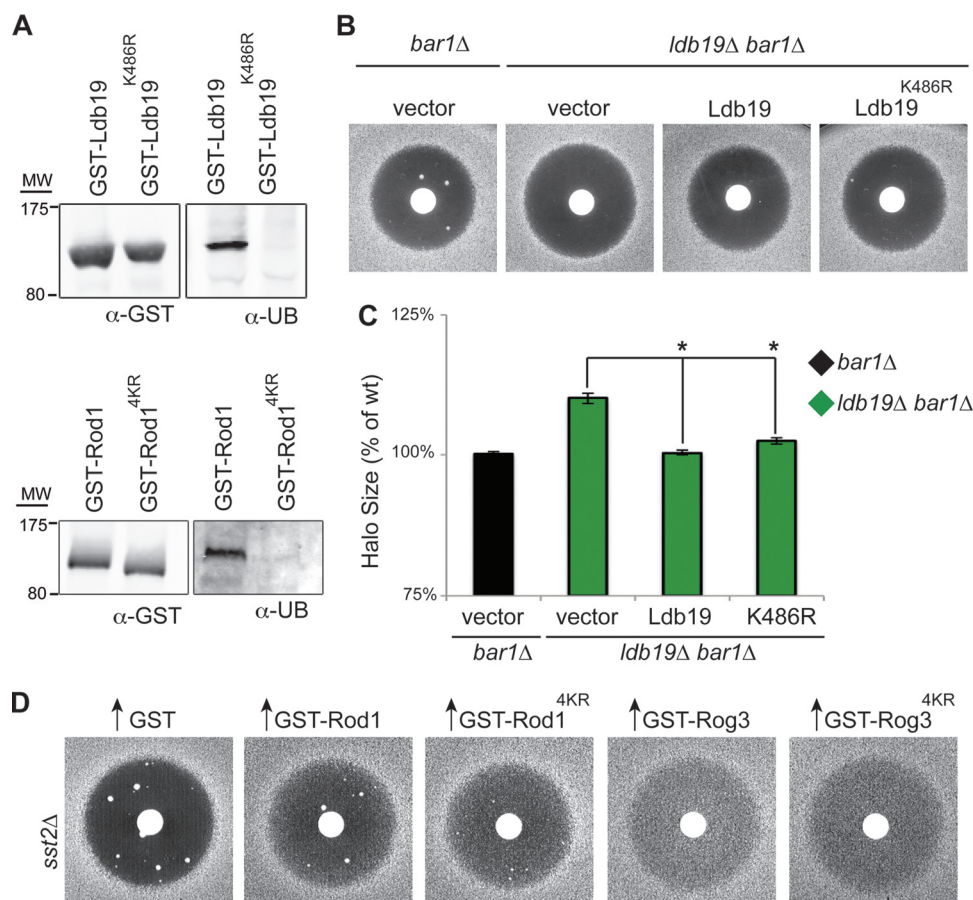


FIG 6 Ubiquitinylation of Ldb19, Rod1, and Rog3 is not required for downregulation of pheromone signaling. (A) For analysis of *in vivo* ubiquitinylation of Ldb19, cultures of a GEV derivative of BJ5459 (JT6743) were grown to mid-exponential phase. Expression of either Ldb19 or the derived K486R substitution mutant (as a GST fusion from the *GAL* promoter) was then induced with β -estradiol for 3 h, and lysates were immediately prepared and analyzed as described for Fig. 4B using the indicated antibodies. For analysis of *in vivo* ubiquitinylation of Rod1, cells were grown to mid-exponential phase in 4% raffinose. Expression of either Rod1 or the derived 4K-to-R mutant (as a GST fusion from the *GAL* promoter) was induced by addition of galactose (2% final concentration) for 3 h and then shifted to dextrose medium (2% final concentration) for 5 min, and lysates were immediately prepared and analyzed as described for Fig. 4B. MW, molecular weight in thousands. (B) Pheromone sensitivity of a *MATa bar1Δ* (JT5915) strain and an isogenic *ldb19Δ bar1Δ* derivative (JT5916) carrying either empty vector (*HIS3*-marked *CEN* plasmid) or the same vector expressing wild-type *LDB19* or the K486R substitution mutant was determined as described in the legend to Fig. 1A, except that the medium was SC-His. (C) Quantification and statistical analysis of the change in halo diameter, determined as described for panel B, from independent replicate experiments ($n = 3$). The average halo diameter for control cells was set at 100%, and the halo sizes for each mutant were normalized to the control. Error bars indicate \pm SEM; *, $P < 0.0001$. (D) Pheromone sensitivity of cultures of *MATa sst2Δ* GEV cells (JT5919) expressing either GST-Rod1 or GST-Rog3, as indicated, or the derived 4K-to-R substitution mutants under the *GAL* promoter on a high-copy-number *URA3*-marked 2 μ m DNA plasmid was determined as described for Fig. 1E. Data from one representative experiment ($n = 3$) are shown.

adaptation assay, overexpression of either wild-type *ROG3* or *ROG3*^{4KR} in *sst2Δ* cells produced turbid halos (Fig. 5D). Thus, the ability of Rog3 to squelch signaling requires neither its Rsp5 interaction (Fig. 5C and E) nor, presumably, its own ubiquitinylation (assuming that, as in Rod1, the 4K-to-R mutations eliminate Rog3 ubiquitinylation).

Ldb19, Rod1, and Rog3 associate with the C-terminal cytosolic tail of Ste2. Extraction of the receptor from membranes requires addition of detergent (21, 110), which might cause misfolding and disrupt protein-protein interactions. Cumulative evidence indicates that one of the most dramatic changes that occurs in the receptor upon pheromone binding *in situ* is a conformational alteration that makes its C-terminal extension (cytosolic tail) more susceptible to attack by exogenously added trypsin (111), to greatly enhanced Yck1- and Yck2-dependent phosphorylation (21, 37, 38, 41), and to overt Rsp5-dependent ubiquitiny-

lation at multiple sites (41, 55). These findings suggest that Ldb19, Rod1, and/or Rog3 interact with this same region of the receptor once it becomes exposed, facilitating Rsp5-dependent modification. Hence, we examined the ability of these α -arrestins to interact with a purified soluble version of the 135-residue C-terminal extension of Ste2 [GST-Ste2(297-431)] bound to beads. Moreover, because we demonstrated (Fig. 6) that ubiquitinylation of Ldb19, Rod1, and Rog3 is not required for their actions *in vivo*, it was not necessary to prepare ubiquitin-decorated versions of each molecule to analyze their ability to associate with the Ste2 tail *in vitro*. Indeed, we found that ³⁵S-labeled Ldb19, Rod1, and Rog3 reproducibly bound better to the C-terminal tail of Ste2 than to the GST control (Fig. 7A and B). Other α -arrestins (e.g., Aly1/Art6) and Art5 (data not shown) did not show any increase above the level of the control (Fig. 7A and B). Moreover, consistent with the fact that its overexpression promoted recovery of *sst2Δ* cells,

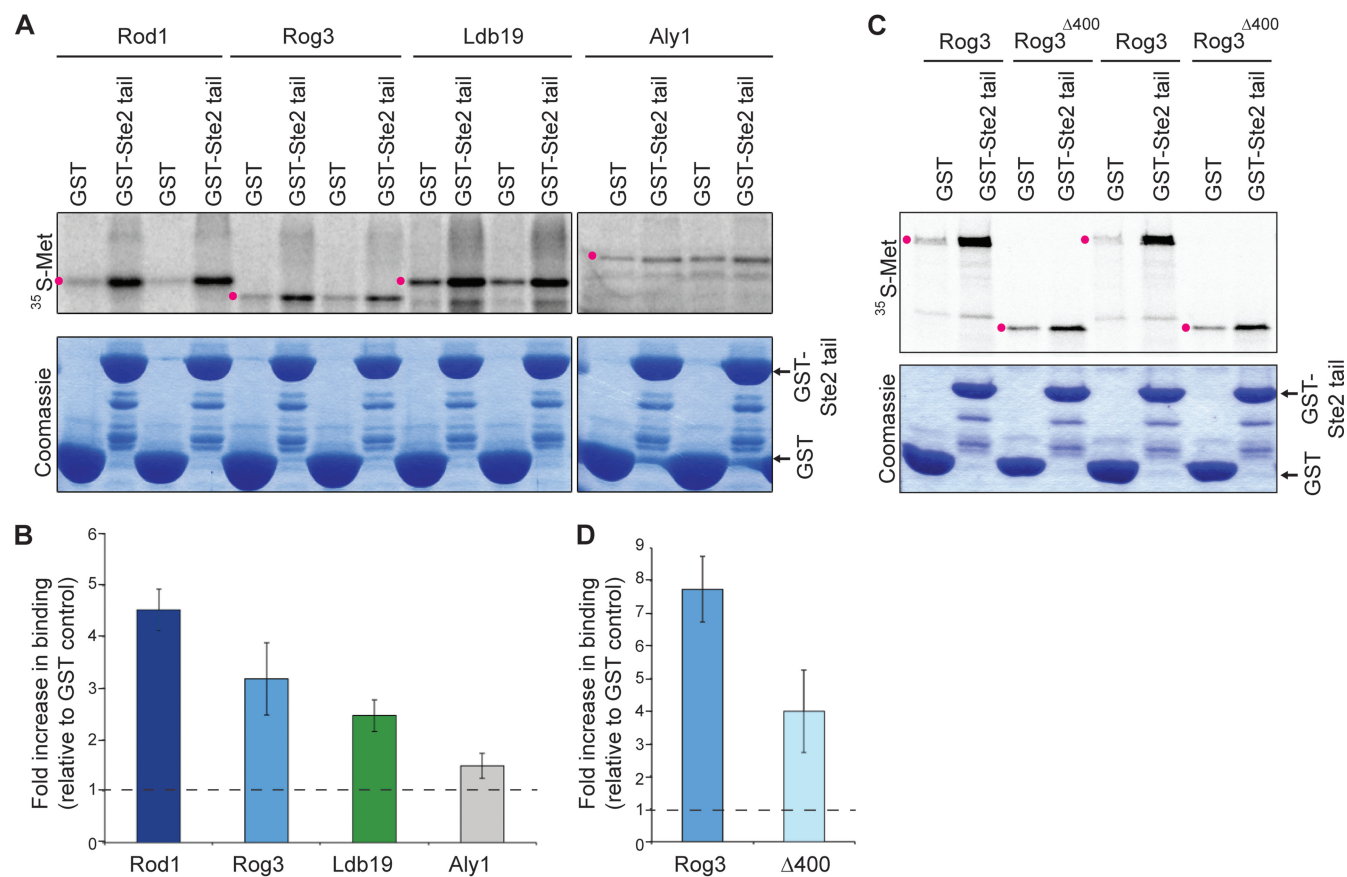


FIG 7 Ldb19, Rod1, and Rog3 bind preferentially to the C-terminal tail of Ste2. (A, lower) GST and GST-Ste2(297-431) (the tail), which was constructed, expressed in *E. coli*, and purified to apparent homogeneity as described previously (21), were used to coat glutathione-agarose beads to an equivalent level. (Upper) In this representative experiment, samples of the beads were incubated in duplicate with equivalent amounts (cpm) of [³⁵S]Met-labeled molecules of the indicated α -arrestins (pink dots) and prepared by coupled *in vitro* transcription and translation. The amount of bound radioactive protein detected was quantified using a phosphorimager as described in Materials and Methods. (B) Average fold increase in the level of radioactivity bound to the GST-Ste2^{tail} construct relative to that bound to the GST control for the indicated α -arrestins in independent replicates ($n = 3$), each performed essentially as described for panel A. Error bars indicate \pm SEM. The dashed line indicates behavior expected for a negative control [i.e., no increase in binding to GST-Ste2(297-431) compared to that of GST alone, yielding a ratio of 1]. (C) Same as described for panel A, except that binding of *in vitro*-transcribed and -translated Rog3 and Rog3^{Δ400} are compared. (D) The average fold increase in binding, determined in panel B for independent replicates ($n = 3$), each performed as described for panel C.

we found that Rog3(Δ 400-733) retained the ability to bind to the Ste2 cytosolic tail (Fig. 7C and D). Thus, Ldb19, Rod1, and Rog3 fulfill all of the requirements of adaptors for the downregulation, modification, and endocytosis of Ste2; they associate specifically with Rsp5, bind preferentially to the C-terminal tail of Ste2, and reduce Ste2 abundance at the cell surface.

Rod1 must be dephosphorylated by calcineurin to promote signal desensitization. Endocytosis of PM nutrient transporters is regulated by metabolic conditions. For example, under glucose-replete conditions, the lactate transporter Jen1 is internalized in a Rod1- and Rsp5-dependent manner (102). Upon glucose limitation, however, Snf1 (yeast AMPK) is activated (112) and phosphorylates Rod1 (113), inhibiting its function. This event allows Jen1 to accumulate in the PM (102). Similarly, we noted during the course of our studies that overexpression of Rod1 on galactose medium, another condition under which Snf1 is active (112), failed to promote adaptation of *sst2* Δ cells (data not shown). These data suggest that Rod1 phosphorylation blocks its association with Ste2. Glc7 (yeast phosphoprotein phosphatase 1) bound to targeting subunit Reg1 has been implicated in the dephosphorylation of

Rod1 required for Jen1 internalization (102). For several reasons, we suspected that the role of Rod1 in promoting pheromone signal desensitization is a feedback mechanism that requires dephosphorylation controlled by Ca^{2+} and the Ca^{2+} -activated phosphatase CN (yeast phosphoprotein phosphatase 2B). First, elevated Ca^{2+} influx is a consequence of pheromone action (114). Second, we showed previously that optimal CN function is required for efficient adaptation after *MATa* cells are exposed to α -factor (115). Third, we demonstrated recently that CN-mediated dephosphorylation of another α -arrestin, Aly1, is required to promote endocytosis of the aspartate and glutamate transporter Dip5 (78). Finally, like Aly1, Rod1 was identified in a global screen for CN substrates and found to be efficiently dephosphorylated by CN *in vitro* (116).

CN is recruited to substrates that contain a conserved docking motif, i.e., PXIXIT and variants thereof (117). Indeed, compared to a GST control, a GFP-tagged derivative of the CN catalytic subunit Cna1 copurified with GST-Rod1 from yeast extracts, whereas an equivalent level of GST-Rod1^{AQAKAA} (in which the sole PXIXIT motif, ⁵⁴⁵PQIKIE⁵⁵⁰, was mutated) exhibited a dra-

matic decrease (>90%) in the amount of Cna1-GFP recovered (Fig. 8A). Consistent with this site being required for efficient CN-dependent dephosphorylation *in vivo*, we found that GST-Rod1^{AQAKAA} resolved into two bands in cells in which CN was activated by exposure to 200 mM CaCl₂, whereas GST-Rod1 migrated as a single band (Fig. 8B, upper). The GST-Rod1^{AQAKAA}-derived species were clearly phosphorylated, because they collapsed to a single faster-mobility band upon treatment with λ phosphatase (Fig. 8B, upper). Likewise, GST-Rod1 also migrated as two distinct bands when the cells were treated with the potent CN-specific inhibitor FK506 (Fig. 8B, upper) or in mutants lacking either the Ca²⁺-binding regulatory subunit (Cnb1) of CN (Fig. 8B, middle) or both of its catalytic subunit isoforms (Cna1 and Cna2) (Fig. 8B, lower). These GST-Rod1-derived bands comigrated with those observed for GST-Rod1^{AQAKAA}. These species also collapsed to the same single faster-mobility species after λ phosphatase treatment (Fig. 8B). These findings demonstrate that efficient dephosphorylation of Rod1 *in vivo* requires CN action.

In agreement with the conclusion that phosphorylation of Rod1 at these CN-sensitive sites blocks its ability to promote desensitization after pheromone response, we observed less turbid halos in the adaptation assay when GST-Rod1^{AQAKAA} was overexpressed in *sst2Δ* cells than with GST-Rod1 (Fig. 8C). Most tellingly, in cells lacking functional CN (either *cnb1Δ* or *cna1Δ cna2Δ* mutants), overexpression of GST-Rod1 was unable to promote any detectable adaptation (Fig. 8D). In contrast, adaptation promoted by GST-Rog3 remained unaffected (Fig. 8D), even though GST-Rod1 and GST-Rog3 were expressed at an equivalent level in both wild-type cells and the CN-deficient mutants (Fig. 8E). We conclude that dephosphorylation mediated by the Ca²⁺-dependent phosphatase CN is essential for Rod1 to downregulate pheromone signaling.

DISCUSSION

It has been presumed that the ligand-induced phosphorylation (37, 38) and Rsp5-dependent ubiquitinylation of multiple Lys residues (41, 42, 55) in the C-terminal cytosolic tail of Ste2, and the ensuing increased rate of receptor endocytosis (33, 34), contributes to signal dampening and recovery from pheromone response. Consistent with this notion, truncations that eliminate the C-terminal tail of Ste2 prevent receptor internalization and result in increased pheromone sensitivity and marked prolongation of pheromone-imposed G₁ arrest (37, 118). However, we now know that the C-terminal tail of Ste2 is also the primary binding site for the RGS protein Sst2; thus, it is required for efficient PM recruitment and function of Sst2 in deactivation of GTP-bound Gpa1 (21). Moreover, absence of Sst2 results in an elevation in pheromone sensitivity and in a sustained pheromone response quite similar to that conferred by receptor C-terminal truncations (37, 118), raising the formal possibility that, compared to its role in tethering Sst2 in the vicinity of its substrate, the C-terminal tail-dependent endocytosis of Ste2 *per se* has little or no function in postpheromone adaptation. Contrary to that viewpoint, the effects of an *sst2Δ* mutation and receptor truncation are somewhat additive, and high-level overexpression of *SST2* can promote recovery of tail-less Ste2 mutants from the effects of pheromone (37, 118).

The findings we describe here support the conclusion that Rsp5-dependent modification and internalization of the receptor contribute to the overall desensitization process. In addition to its

role in recovery from pheromone, receptor endocytosis may contribute to generating the receptor distribution for the polarized chemotropic growth that occurs during mating (51). Most importantly, our work answers previously unresolved questions about how Ste2 is recognized by Rsp5 to promote both its basal and ligand-induced endocytosis. Given that Rsp5 associates with the PM via its N-terminal lipid-binding C2 domain (119) but interacts with the substrates it modifies via the binding of its three tandem WW domains (103) to PPXY motifs in those targets (57, 120), it was a conundrum as to how Rsp5 recognized Ste2 as a substrate, since this receptor lacks any sequence elements resembling PPXY. The discovery that members of the α -arrestin family of adaptor proteins serve as intermediaries to recruit Rsp5 to nutrient permeases that also lack endogenous PPXY motifs suggested that members of the α -arrestin class of proteins function as the molecular matchmakers for delivering Rsp5 to Ste2.

Indeed, as documented here, three of the 14 known yeast α -arrestins contribute to signal downregulation at the receptor level, as judged by multiple independent criteria. First, lack of either Ldb19 or the paralogous pair Rod1 and Rog3 increases pheromone sensitivity, even in cells that possess all of the other demonstrated mechanisms for recovery from pheromone response. Second, simultaneous absence of Ldb19, Rod1, and Rog3 results in even greater pheromone sensitivity, comparable to that of a cell in which 9 α -arrestins (including Ldb19, Rod1, and Rog3) are absent. Third, among all of the α -arrestins tested, only Rod1, Rog3, and Ldb19 exhibited preferential binding to the site of Rsp5-dependent modification in Ste2, its cytosolic C-terminal tail. Phosphorylation of purified GST-Ste2^{tail} with recombinant casein kinase I neither enhanced nor inhibited binding of Ldb19, Rod1, or Rog3 (A. F. O'Donnell, unpublished results), indicating that, rather than creating epitopes for recruitment of these adaptors, phosphorylation simply assists in locking the tail in a solvent-accessible state after it is exposed by the pheromone-induced change in receptor conformation. Fourth, in cells expressing fluorescent derivatives of Ste2, PM accumulation of the receptor occurred in *ldb19Δ* cells, in *rod1Δ rog3Δ* cells, and especially in *ldb19Δ rod1Δ rog3Δ* (and *9arrΔ*) cells, and it was not observed in control cells or in any other single or multiple α -arrestin deletion mutants.

The bulk of either mCherry- or eGFP-tagged Ste2 resided in the vacuole under all circumstances. This behavior suggests that a large fraction of these chimeras can be recognized as incorrectly folded (and/or improperly glycosylated) and shunted to the vacuole via the Golgi body-to-endosome quality-control pathway (121, 122) more efficiently than they are delivered from the Golgi compartment to the PM. Therefore, the amount of these fluorescently tagged proteins that is properly folded and inserted into the PM may be a minority of the total synthesized. Moreover, the Golgi body-to-endosome shunt is known to depend on decoration of cargo by ubiquitin (123), making it problematic to analyze the change in modification state of our Ste2 constructs due solely to the presence or absence of Ldb19 and/or Rod1 and Rog3. Nevertheless, the observed increase in the amount of tagged Ste2 at the PM in *ldb19Δ*, *rod1Δ rog3Δ*, and *ldb19Δ rod1Δ rog3Δ* cells is consistent with their role in mediating the Rsp5-dependent endocytosis of the receptor.

Our results also show that the three α -arrestins contribute to Ste2 downregulation in discrete ways (Fig. 9). First, although paralogs Rod1 and Rog3 may have some overlapping role (be-

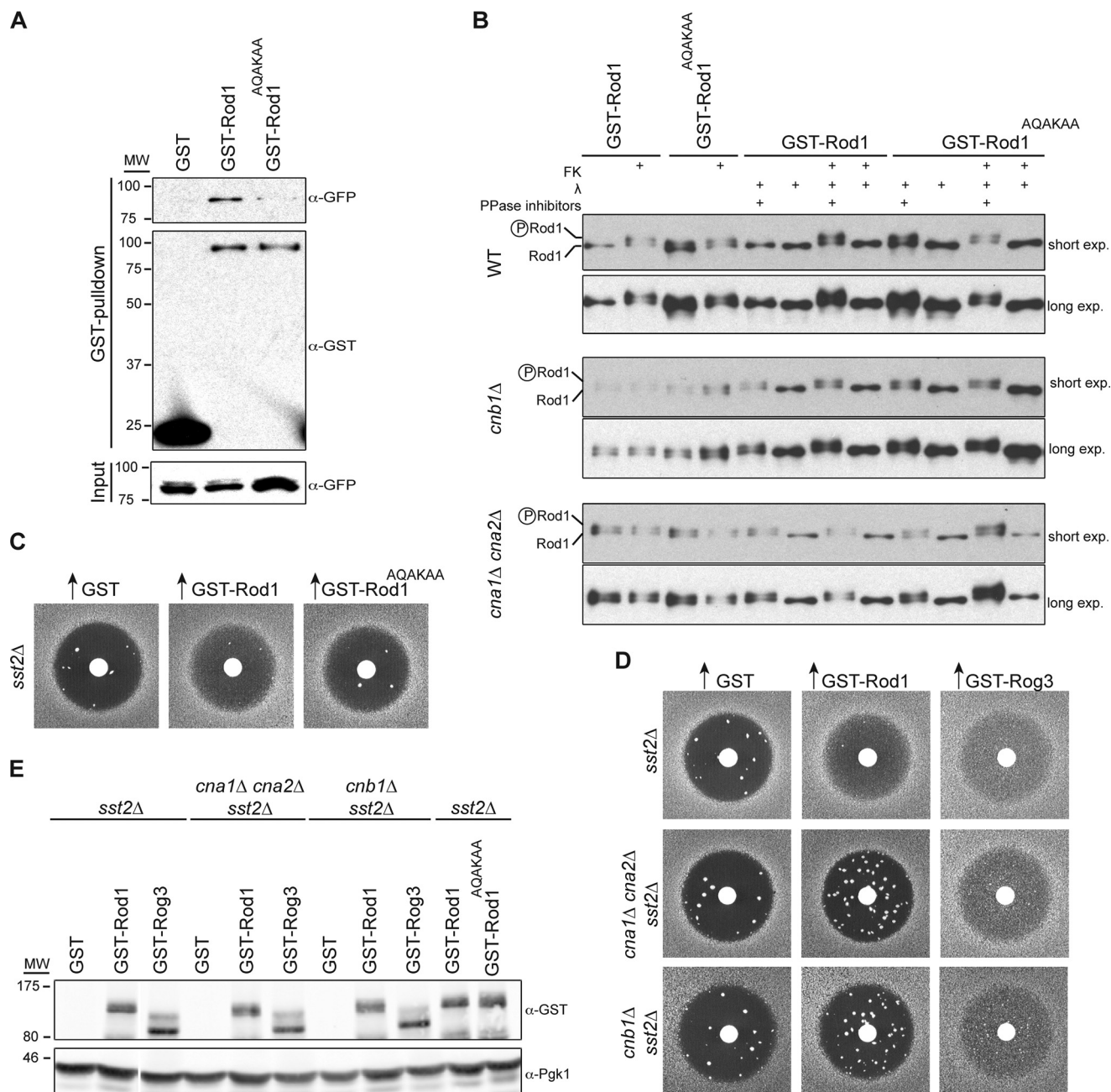


FIG 8 Rod1-mediated desensitization requires calcineurin-dependent dephosphorylation. (A) A single PXIXIT motif mediates CN-Rod1 interaction. Cultures of strain JRY11, which produces Cna1-GFP from the native *CNA1* promoter at the endogenous *CNA1* locus on chromosome XII, and also expressing, as indicated, either GST alone, GST-Rod1, or GST-Rod1^{AQAKAA}, were grown to mid-exponential phase, harvested, and lysed, and proteins in the resulting extracts were captured on glutathione-agarose beads, resolved by SDS-PAGE, and analyzed with the indicated antibodies. MW, molecular weight in thousands. (B) Rod1 is phosphorylated at CN-sensitive sites. Cultures of strain BY4741 (WT) or otherwise isogenic *cnb1Δ* (BY4741 *cnb1Δ*) and *cna1Δ cna2Δ* (JT5574) derivatives, as indicated, expressing either GST-Rod1 or GST-Rod1^{AQAKAA} were grown to mid-exponential phase and stimulated with 200 mM CaCl₂ to activate CN in either the absence or presence (+) of the CN inhibitor FK506 (FK). After harvesting and lysis, proteins in the resulting extracts were purified by capture on glutathione-agarose beads (lanes 1 to 4). Samples of the material shown in lanes 1 to 4 then were either left untreated or were treated (+) with lambda phosphatase (λ) in either the absence or presence (+) of phosphatase inhibitors (PPase inhibitors), and the resulting products were separated under SDS-PAGE conditions that permit resolution of phospho-isomers and analyzed with anti-GST antibodies. exp., exposure. (C) Lack of CN binding reduces Rod1-mediated adaptation. Pheromone sensitivity of *MATa sst2Δ GEV* (JT5919) cells expressing either GST-Rod1 or GST-Rod1^{AQAKAA}, as indicated, under the *GAL* promoter on a high-copy-number *URA3*-marked 2 μm DNA plasmid was determined as described for Fig. 1E. Data from one representative experiment (*n* = 3) are shown. (D) Absence of CN eliminates Rod1-mediated adaptation but not Rog3-mediated adaptation. Pheromone sensitivity of cultures of *MATa sst2Δ GEV* (JT5919) cells and isogenic *cnb1Δ* (JT6694) and *cna1Δ cna2Δ* (JT6695) derivatives, as indicated, expressing either GST-Rod1 or GST-Rog3 under the *GAL* promoter on a high-copy-number *URA3*-marked 2 μm DNA plasmid was determined as described for Fig. 1E. Data from one representative experiment (*n* = 3) are shown. (E) Confirmation of protein expression. Whole-cell extracts of the cells used in panels C and D were prepared, resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Here, phospho-isomers were not separated, because different SDS-PAGE conditions were used. Data from one representative experiment (*n* = 3) are shown.

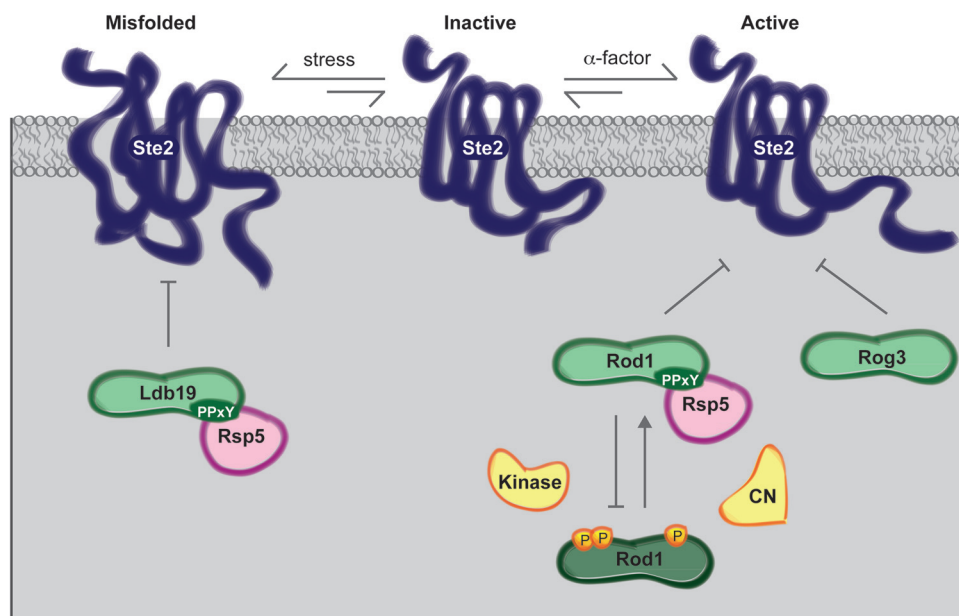


FIG 9 Distinct mechanisms of Ste2 downregulation by the α -arrestins Ldb19, Rod1, and Rog3. The α -factor receptor (Ste2), a polytopic integral membrane protein, exists primarily in three conformational states. In naïve cells, Ste2 undergoes spontaneous stochastic dissociation from its cognate heterotrimeric G protein (not shown for clarity) at a certain rate and thereby becomes destabilized. When it does so, it may misfold. Current evidence suggests that Ldb19/Art1 has a primary role in a PM quality-control pathway that mediates Rsp5-dependent ubiquitinylation and endocytic removal of such misfolded PM proteins. In the presence of α -factor, Ste2 undergoes a ligand-induced conformational change that activates and dissociates its cognate G protein; however, in this case, the receptor is stabilized by bound pheromone. Because Rog3-imposed inhibition of pheromone signaling does not obligatorily require its association with or modification by Rsp5, it may act similarly to classical arrestin or β -arrestin by binding to the C-terminal tail of the receptor and sterically preventing additional rounds of G-protein activation by the pheromone-bound receptor. Later during response to pheromone, Ca^{2+} influx will stimulate the CN-dependent dephosphorylation of Rod1, making Rod1 competent to mediate Rsp5-dependent ubiquitinylation and endocytic removal of the pheromone-bound receptor, a prime example of a stimulus-induced, late-stage, negative-feedback control.

cause a detectable phenotype was found only for the *rod1 Δ rog3 Δ* double mutant), the loss of Ldb19 and absence of Rod1 and Rog3 had additive effects. Second, like Ldb19, the ability of Rod1 to promote adaptation requires Rsp5 binding, whereas the function of Rog3 in adaptation does not. Third, in the absence of CN-dependent dephosphorylation, Rod1 is unable to promote adaptation, whereas Rog3 is not subject to CN-mediated regulation. The most parsimonious interpretation of our collective findings is that the three different α -arrestins act on different states of the receptor and do so under different conditions (Fig. 9).

Once inserted into the PM, Ste2, like any GPCR, will exist primarily in three conformational states. Even in the absence of pheromone stimulation, Ste2 undergoes spontaneous dissociation from its cognate heterotrimeric G protein. In the absence of the stabilization conferred by receptor-G-protein interaction, Ste2 can misfold. It was shown recently that Ldb19/Art1 is essential for the Rsp5-dependent ubiquitinylation and internalization of the lysine permease Lyp1, but only after it had been subjected to heat stress (124). Thus, in the same way, the effects we observed in *ldb19 Δ* cells may be explained if Ldb19 has a similar role in PM quality control in that it mediates Rsp5-dependent ubiquitinylation and endocytic removal only of misfolded Ste2. Consistent with this view, Ldb19 bound less avidly than did either Rod1 or Rog3 to the isolated Ste2 tail *in vitro*, presumably because Ldb19 recognizes an additional determinant only accessible in a misfolded receptor. For example, because native Ste2 functions as a dimer (125–127), perhaps Ldb19 associates with the tail and also a site exposed in a monomer when the dimer dissociates. In any

event, a role in removal of misfolded molecules may explain why overexpression of Ldb19 was unable to enhance the rate of recovery in the adaptation assay, which is conducted under conditions where the properly folded state of the receptor is stabilized by ligand binding. In the absence of Ldb19, unfolded receptor is not removed from the cell surface, explaining the increase in Ste2-mCherry and Ste2-GFP observed in *ldb19 Δ* cells. Moreover, since misfolded receptor is not removed from *ldb19 Δ* cells, it has a chance to refold and recouple to the G protein, raising the concentration of receptor competent for signaling in the first place and explaining the modest increase in pheromone sensitivity displayed by *ldb19 Δ* cells.

When *MATa* cells are exposed to α -factor, Ste2 undergoes a ligand-induced conformational change that activates and dissociates its cognate G protein; however, in contrast to when the G protein stochastically dissociates from the naïve receptor in untreated cells, activated receptor is stabilized by bound pheromone. Because Rog3 potently stimulates desensitization and does not obligatorily require its association with or modification by Rsp5 to do so, it may act in a manner similar to that of retinal arrestin or β -arrestin (128). Specifically, by binding to the C-terminal tail of the receptor, Rog3 may sterically prevent the pheromone-bound receptor from catalyzing additional rounds of G-protein activation. Indeed, just like mammalian arrestin and β -arrestin, which lack PPXY motifs and have no long C-terminal extension (59, 60), the arrestin fold domain at the N terminus of Rog3 is sufficient to promote adaptation. Although technically challenging, it will be important to determine in future experiments whether Rog3

competes with Gpa1 for receptor binding. Alternatively, Rog3 may promote the clathrin- and Sla1-dependent, but ubiquitin-independent, route of endocytosis that has been defined for membrane proteins that contain an exposed NPFX₁₋₂D motif (129–131). Ste2 contains a very similar sequence, ³⁹²GPFAD³⁹⁶, in its C-terminal tail. On the other hand, although Rog3 does not need to recruit Rsp5 to execute its role in squelching pheromone signaling, it does possess P/VPXY motifs competent to bind Rsp5. Hence, normally, Rog3 may both interfere with receptor-G-protein recoupling and mediate Rsp5-dependent ubiquitinylation, thereby enhancing the efficiency of Ste2 capture by the components of the endocytic machinery that recognize ubiquitinated cargo (132). This dual function may explain why Rog3 appears more efficacious than Rod1 in promoting adaptation when overexpressed. Moreover, Ste2 is the first target of Rog3 identified.

Our results indicate that Rod1 is a component of a negative feedback loop that ensures complete receptor clearance after a MATa cell has committed to a productive pheromone response. One hallmark of the later stages of pheromone response is a robust influx of Ca²⁺ (133, 134). This rise in intracellular Ca²⁺ is sufficient to activate the Ca²⁺-dependent phosphatase calcineurin (135). As we have demonstrated here, CN-mediated dephosphorylation makes Rod1 competent to stimulate adaptation after MATa cells are exposed to α -factor (Fig. 9). In contrast, loss of CN activity did not prevent Rog3-promoted adaptation to pheromone, demonstrating that regulation by CN is specific to Rod1. Indeed, the PXIXIT motif in Rod1 is not conserved in Rog3. Along with Aly1 (78), Rod1 is now the second yeast α -arrestin shown to be under CN regulation. Also, in *Caenorhabditis elegans*, the function of α -arrestin CNP-1/ArrD-17 requires CN-mediated dephosphorylation (136), suggesting that CN control of α -arrestin dephosphorylation is a conserved regulatory mechanism.

Snf1 negatively regulates Rod1 function in response to glucose limitation, thereby preventing endocytosis of the lactate permease Jen1 (102, 113). Similarly, AMPK phosphorylates and promotes degradation of the mammalian α -arrestin family member TXNIP, thereby increasing glucose uptake by preventing TXNIP-mediated downregulation of the glucose transporter GLUT1 (137). Moreover, in glucose control of Rod1 action on Jen1, Reg1-bound Glc7 seems to be responsible for Rod1 dephosphorylation (64) and likely also prevents Snf1-mediated phosphorylation of Rod1 by deactivating Snf1 itself (138). However, we observed a requirement for CN-dependent dephosphorylation for Rod1 action on Ste2 on glucose-rich medium, a condition under which Snf1 is not activated (112). These observations raised two important points. First, Snf1 cannot be the only protein kinase responsible for phosphorylating and inhibiting Rod1. In fact, phosphorylation seems to be a general mechanism for blocking the endocytic action of other α -arrestin family members (78, 108, 139). Second, it is clear, at least in the case of Rod1, that the same α -arrestin is being subjected to differential phospho-regulation as a means to control endocytosis of different targets in response to distinct stimuli.

Previous reports provide evidence that ubiquitinylation of Ldb19 (57) and Rod1 (102) is necessary for their function in internalizing other cargoes. We found, however, that ubiquitinylation of Ldb19, Rod1, and Rog3 themselves was dispensable for negative regulation of Ste2-initiated signaling. Moreover, compared to the wild-type proteins, we observed only modest increases in the steady-state level of nonubiquitinylatable (PPXY-less and K-to-R) variants of these three α -arrestins, suggesting

that their Rsp5-mediated modification does not trigger rapid proteasome-mediated degradation. Indeed, Rsp5 is known to install K63-linked polyubiquitin chains on target Lys residues (140), and binding of accessory proteins, such as ESCRT-0 (141) and ESCRT-1 (109), blocks K63-linked chain recognition by proteasomes (141) or K63-linked chain formation on substrates (142). Moreover, paring back of the K63-linked chains by cellular deubiquitinating enzymes leaves monoubiquitinated Lys residues (143), which are poorly recognized by the proteasome (144). Although ubiquitinylation of Ldb19, Rod1, and Rog3 is not required for their function, decoration with ubiquitin might prevent Rsp5 binding. If so, ubiquitinylation of these α -arrestins themselves would provide a built-in delay timer that, after an appropriate extent of modification, eventually dissociates Rsp5 and thereby recycles this E3. Alternatively, like phosphorylation, ubiquitinylation may be yet another means to control differentially the interaction of the same α -arrestin with different targets in response to discrete signals.

Presumably, defects in *LDB19*, and especially in *ROD1* and *ROG3*, were not identified in standard screens for loss-of-function mutations that confer elevated pheromone sensitivity because of their overlapping functions. Indeed, in the case of *ROD1* and *ROG3*, we observed a detectable phenotype only in the *rod1* Δ *rog3* Δ double mutant. One reason the effects of these mutations appears modest is that the level of these α -arrestins is quite low (Ldb19, 295 per cell; Rod1, 386 per cell [Rog3 was not reported] [145]) compared to the number of receptors on the surface of a MATa cell, \sim 8,000 per cell (32, 125, 146). Thus, this difference in stoichiometry may explain why it has been technically difficult to observe colocalization of these molecules with Ste2 under normal cellular conditions (C. Alvaro, unpublished observations). In contrast, colocalization of the much more abundant Sst2 (5,980 molecules per cell [145]) with Ste2 was readily observed (21). Indeed, colocalization of Ldb19 (57) and Rod1 (102) with their respective cargoes has not been demonstrated, even when overexpressed (C. Alvaro, unpublished observations), perhaps indicating the transient nature of α -arrestin-target interaction. Nonetheless, when the quantity of either Rod1 or Rog3 was elevated, the adaptation assay revealed that the ability of these α -arrestins to squelch signaling by the ligand-bound form of the receptor is actually quite potent.

In addition to Ste2, it is formally possible that Ldb19, Rod1, and/or Rog3 mediates the Rsp5-dependent modification of other factors that might lead to downregulation of a pheromone-induced signal. One study indicates that the G α subunit (Gpa1) of the Ste2-associated heterotrimeric G protein undergoes Rsp5-mediated mono- and polyubiquitinylation, which reportedly downregulates the amount of Gpa1 at the PM by diverting it to the vacuole or to the proteasome, respectively (147). Because Gpa1 holds the G $\beta\gamma$ (Ste4-Ste18) complex in check and prevents signal propagation, loss of any factor that contributes to Rsp5-dependent modification of Gpa1 would stabilize Gpa1, increase Gpa1 abundance at the PM, and make cells less sensitive to pheromone. Hence, Ldb19, Rod1, and Rog3 cannot be involved in the reported Rsp5-dependent modification of Gpa1, because as we have documented here, loss of these α -arrestins makes cells more sensitive to pheromone action. Similarly, it has been noted that Ste4 (G β) becomes ubiquitinated in an Rsp5-dependent manner on Lys340, but the presence or absence of this modification does not affect the rate of turnover of Ste4 and, unlike loss of Ldb19, Rod1,

and Rog3, does not affect the magnitude or duration of pheromone-induced Fus3 activation (148). Hence, Ldb19, Rod1, and Rog3 cannot negatively regulate pheromone responses by being responsible for mediating the reported Rsp5-dependent modification of Ste4.

Taken together, our findings indicate that, in *S. cerevisiae*, α -arrestins Rod1, Rog3, and Ldb19 are negative regulators of the α -factor receptor (Ste2) in *MATa* cells. In yeast, there are other GPCRs, including the a-factor receptor (Ste3) in *MATa* cells (149), an apparent glucose sensor (Gpr1) (150), and an alkaline pH sensor (Rim21) (109). It will be important to determine which α -arrestins regulate these GPCRs and whether, as observed for nutrient permeases (58, 124), there is both specificity and redundancy in which α -arrestins modulate these targets, as we have found for Ste2.

Since the time we first reported our initial observations about the apparent functions of Ldb19, Rod1, and Rog3 in contributing to downregulation of GPCR-initiated signaling in yeast (151), members of the α -arrestin family in animal cells have been implicated in interacting with, modifying, and/or promoting the desensitization and endocytosis of several classes of GPCRs (61, 152–155). However, it has been reported previously that β -arrestins fulfill this role (156, 157); furthermore, the molecular mechanisms by which mammalian α -arrestins may contribute to downregulation of GPCR signaling is currently in dispute (152, 154). Because *S. cerevisiae* lacks any β -arrestin homolog, our results indicate that α -arrestins alone are capable of promoting GPCR internalization. Thus, our studies in a model organism have helped to resolve an important biological question. Therefore, α -arrestin-mediated downregulation of GPCR-initiated signaling is likely a conserved regulatory mechanism in eukaryotes. In *S. cerevisiae*, there are currently 14 documented α -arrestin members (60, 108, 158), whereas, to date, only eight (ArrDC1 to ArrDC5, TXNIP, and possibly DSCR3 and RGP1) are recognized in animal cells (159). Moreover, the number of mammalian GPCRs is very large. Hence, it is highly likely that many more mammalian α -arrestins remain to be identified and characterized and their targets delineated.

ACKNOWLEDGMENTS

This work was supported by NIH CBMS predoctoral training grant GM07232 (to C.G.A.), by NIH CBMS predoctoral training grant GM07276 (to A.G.), by NIH R01 research grant GM48729 (to M.S.C.), by NSF grant MCB1024818 (to B.W.), and by NIH R01 research grant GM21841 (to J.T.). For studies at UC Berkeley, A.F.O. was supported by GM21841. For studies at the University of Pittsburgh, work was supported by NIH R01 research grant GM75061 (to J.L.B.) and NIH R01 research grant DA014204 (to Alexander Sorkin) and by development funds from the Department of Cell Biology, University of Pittsburgh School of Medicine.

We thank Hugh Pelham (LMB, MRC, Cambridge, United Kingdom) for the *9arrΔ* strain, Jon M. Huibregtse (University of Texas, Austin) for GST-Rsp5 expression constructs, Mary Matyskiela and Andreas Martin (UC Berkeley) for the gift of purified Uba1 (E1) and Ubc1 (E2) and assistance with GST-Rsp5 purification, Greg Barton (UC Berkeley) for FlowJo software and assistance with FACS analysis, and Scott Emr (Cornell University) and Sébastien Léon (Institut Jacques Monod, Paris, France) for helpful discussions.

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