

Differential Phosphorylation Provides a Switch to Control How α -Arrestin Rod1 Down-regulates Mating Pheromone Response in *Saccharomyces cerevisiae*

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ABSTRACT G-protein-coupled receptors (GPCRs) are integral membrane proteins that initiate stimulus-dependent activation of cognate heterotrimeric G-proteins, triggering ensuing downstream cellular responses. Tight regulation of GPCR-evoked pathways is required because prolonged stimulation can be detrimental to an organism. *Ste2*, a GPCR in *Saccharomyces cerevisiae* that mediates response of *MATa* haploids to the peptide mating pheromone α -factor, is down-regulated by both constitutive and agonist-induced endocytosis. Efficient agonist-stimulated internalization of *Ste2* requires its association with an adaptor protein, the α -arrestin *Rod1/Art4*, which recruits the HECT-domain ubiquitin ligase *Rsp5*, allowing for ubiquitylation of the C-terminal tail of the receptor and its engagement by the clathrin-dependent endocytic machinery. We previously showed that dephosphorylation of *Rod1* by calcineurin (phosphoprotein phosphatase 2B) is required for optimal *Rod1* function in *Ste2* down-regulation. We show here that negative regulation of *Rod1* by phosphorylation is mediated by two distinct stress-activated protein kinases, *Snf1/AMPK* and *Ypk1/SGK1*, and demonstrate both *in vitro* and *in vivo* that this phospho-regulation impedes the ability of *Rod1* to promote mating pathway desensitization. These studies also revealed that, in the absence of its phosphorylation, *Rod1* can promote adaptation independently of *Rsp5*-mediated receptor ubiquitylation, consistent with recent evidence that α -arrestins can contribute to cargo recognition by both clathrin-dependent and clathrin-independent mechanisms. However, in cells lacking a component (formin *Bni1*) required for clathrin-independent entry, *Rod1* derivatives that are largely unphosphorylated and unable to associate with *Rsp5* still promote efficient adaptation, indicating a third mechanism by which this α -arrestin promotes desensitization of the pheromone-response pathway.

KEYWORDS mating pheromone response; adaptation; desensitization; down-regulation; endocytosis

A cell must adapt rapidly to external stimuli and other changes in its environment. One mechanism to achieve an appropriate response is through remodeling of the repertoire of integral membrane proteins in the plasma membrane (PM), including receptors, channels, permeases, and other transporters. These transmembrane proteins are often shuttled

between different cellular compartments in response to specific stimuli. This trafficking, especially endocytosis to remove these molecules from the PM, is controlled, in all cases examined, by regulated ubiquitylation of the target protein (Horák 2003; Dupré *et al.* 2004; Nikko and Pelham 2009; Lauwers *et al.* 2010; Zhao *et al.* 2013; Crapeau *et al.* 2014; Ghadder *et al.* 2014).

In eukaryotes, G-protein-coupled receptors (GPCRs) are the most abundant class of cell-surface receptors (Granier and Kobilka 2012; Katritch *et al.* 2013). Internalization of a GPCR plays an important role in both rapid and long-term desensitization after exposure of a cell to the cognate agonist (Marchese and Trejo 2013; Irannejad *et al.* 2015). Aberrant GPCR signaling and dysregulation have been implicated in many pathophysiological conditions, including cancers, asthma, hypertension, neurological disorders, and autoimmune diseases

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(O'Hayre *et al.* 2014; West and Hanyaloglu 2015). For these reasons, GPCRs are the targets of the majority of clinically used pharmaceuticals (Shoichet and Kobilka 2012; Zhang and Xie 2012; Garland 2013). A model system that has served as a very informative experimental paradigm for investigating GPCR-initiated signaling and its regulation are the receptors in budding yeast (*Saccharomyces cerevisiae*) that mediate its response to peptide mating pheromones (Hao *et al.* 2007; Merlini *et al.* 2013).

It has been amply demonstrated that both basal and agonist-induced internalization of *Ste2* (the GPCR on *MATa* cells that binds the mating pheromone α -factor) and *Ste3* (the GPCR on *MAT α* cells that binds the mating pheromone **a**-factor) requires ubiquitinylation on Lys residues in their cytosolic tails and that *Rsp5* (mammalian ortholog is Nedd4L) is the ubiquitin ligase (E3) responsible for this modification (Dunn and Hicke 2001; Ballon *et al.* 2006; Rotin and Kumar 2009). *Rsp5* catalyzes formation of K63-linked polyubiquitin chains on its substrates (Galan and Haguenaer-Tsapis 1997; Kim and Huibregtse 2009; Lauwers *et al.* 2009), leading to their recruitment into clathrin-coated pits and internalization (Weinberg and Drubin 2012; Myers and Payne 2013). *Rsp5* associates via its WW domains with PPxY motifs (and variants thereof) in its targets. However, recruitment to many such targets is not direct, but mediated instead by intermediary “adaptor” proteins, and paramount among these molecular matchmakers are the α -arrestins (Lin *et al.* 2008; Léon and Haguenaer-Tsapis 2009; Nikko and Pelham 2009), a family of proteins found in all eukaryotes from yeast to humans (Alvarez 2008; Aubry and Klein 2013). In *S. cerevisiae*, these adaptors have been dubbed Art (for “Arrestin-Related Trafficking”) proteins (Lin *et al.* 2008), whereas in animal cells these are termed ARRDC (for “Arrestin-Domain-Containing”) proteins (Aubry and Klein 2013). In general, in these molecules, an arrestin fold (Aubry *et al.* 2009) situated near their N-terminal end mediates interaction with the target (Kang *et al.* 2015a,b), and PPxY motifs located in their C-terminal region associate with a WW domain-containing HECT-type E3 (Rotin and Kumar 2009).

The *S. cerevisiae* genome encodes 14 recognized α -arrestins, most of which have been implicated in endocytosis and trafficking of various nutrient permeases (Lin *et al.* 2008; Nikko and Pelham 2009; O'Donnell *et al.* 2010; Becuwe *et al.* 2012; Merhi and Andre 2012; O'Donnell *et al.* 2015). We demonstrated recently that specific α -arrestins also control internalization of both *Ste2* (Alvaro *et al.* 2014) and *Ste3* (Prosser *et al.* 2015). In both yeast and mammalian cells, the types of integral PM proteins greatly outnumber the α -arrestins present; hence, there is promiscuity in these interactions; that is, a given α -arrestin can have more than one target. However, in several respects, there is also considerable specificity: (i) most cargo are the target of several α -arrestins, but far from all (Lin *et al.* 2008; Nikko and Pelham 2009; Lauwers *et al.* 2010; Alvaro *et al.* 2014; Prosser *et al.* 2015); (ii) rapid internalization of a given cargo is triggered only in response to a specific stimulus and, as a result, often engages only one

or just a few α -arrestins (Becuwe *et al.* 2012; O'Donnell *et al.* 2013; Zhao *et al.* 2013; Crapeau *et al.* 2014; Ghaddar *et al.* 2014; O'Donnell *et al.* 2015); and (iii) the function of an α -arrestin is often negatively regulated by phosphorylation (Shinoda and Kikuchi 2007; MacGurn *et al.* 2011; Becuwe *et al.* 2012; Jee *et al.* 2012; Merhi and Andre 2012; O'Donnell *et al.* 2013; Alvaro *et al.* 2014; Herrador *et al.* 2015). Arrestin phosphorylation raises important questions about what protein kinases are involved in these control circuits and under what conditions, and how such modifications affect the ability of an α -arrestin to promote internalization of its specific PM protein targets.

We have shown (Alvaro *et al.* 2014) that, in addition to all the other previously known mechanisms for down-regulating the mating pathway (Dohlman and Thorner 2001), three α -arrestins specifically contribute to desensitization of the pheromone response in *MATa* cells by mediating internalization of *Ste2*. *Ldb19/Art1* participates mainly in basal *Rsp5*-dependent endocytosis of *Ste2* (*i.e.*, in the absence of pheromone), most likely through recognition of misfolded forms of the receptor, consistent with other evidence that this α -arrestin primarily serves a “quality control” function (Zhao *et al.* 2013). By contrast, *Rod1/Art4* and its paralog *Rog3/Art7*, promote *Rsp5*-dependent endocytosis of pheromone-bound receptor; however, *Rod1* function in *Ste2* down-regulation obligatorily required its association with *Rsp5*, whereas forms of *Rog3* unable to associate with *Rsp5* were able to promote adaptation. Conversely, the ability of *Rod1* to promote adaptation required its dephosphorylation by the Ca^{2+} /calmodulin-stimulated phosphoprotein phosphatase calcineurin, whereas *Rog3* did not. These findings focused our attention on the underlying mechanisms involved in phospho-regulation of *Rod1*. As described here, we identified two stress-responsive protein kinases that phosphorylate *Rod1 in vivo* and delineated the sites at which they exert their regulatory effect. Our studies also reveal that, in the absence of its phosphorylation, *Rod1* can, like *Rog3*, also promote adaptation in an *Rsp5*-independent manner, suggesting that, in addition to negative regulation, phosphorylation may serve as a switch to control how *Rod1* down-regulates mating pheromone response.

Materials and Methods

Strains and growth conditions

Yeast strains (Table 1) were grown at 30° 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 (Sherman *et al.* 1986). Standard genetic methods were used for strain construction (Amberg *et al.* 2005).

Plasmids

Plasmids (Table 2) were constructed using standard procedures (Green and Sambrook 2012a,b). Briefly, DNA

Table 1 Yeast strains used in this study

Strain	Genotype	Source
<i>sst2Δ</i> GEV (JT5919)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 sst2Δ::SpHIS5 leu2Δ0::GEV::NatMX</i>	Alvaro et al. (2014)
<i>snf1Δ sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 snf1Δ::KanMX4</i>	This study
<i>kin1Δ sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 kin1Δ::KanMX4</i>	This study
<i>kin2Δ sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 kin2Δ::KanMX4</i>	This study
<i>kin4Δ sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 kin4Δ::KanMX4</i>	This study
<i>kcc4Δ sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 kcc4Δ::KanMX4</i>	This study
<i>hsl1Δ sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 hsl1Δ::KanMX4</i>	This study
<i>frk1Δ sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 frk1Δ::KanMX4</i>	This study
<i>gin4Δ sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 gin4Δ::KanMX4</i>	This study
<i>cnb1Δ sst2Δ</i> GEV (JT6694)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 cnb1Δ::KanMX4</i>	Alvaro et al. (2014)
<i>cna1Δ cna2Δ sst2Δ</i> GEV (JT6695)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 cna1Δ::KanMX4</i>	Alvaro et al. (2014)
BJ5459 GEV (JT6743)	<i>MATa ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4Δ::HIS3 prb1Δ1.6R can1 GAL leu2Δ1::GEV::NatMX</i>	Alvaro et al. (2014)
<i>rod1Δ rog3Δ ldb19Δ sst2Δ</i> GEV (JT6716)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rod1Δ::KanMX4 rog3Δ::KanMX4 ldb19Δ::NatMX sst2Δ::SpHIS5</i>	Alvaro et al. (2014)
<i>STE2 sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 STE2::HPH</i>	This study
<i>STE2^{7KR} sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 STE2^{7KR}::HPH</i>	This study
<i>STE2^{Δ296} sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 STE2^{Δ296}::HPH</i>	This study
<i>STE2-mCherry sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-mCherry::URA3 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5</i>	This study
<i>STE2^{7KR}-mCherry sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2^{7KR}-mCherry::URA3 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5</i>	This study
<i>STE2^{7KR}-mCherry sst2Δ</i> GEV ^b	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2^{7KR}-mCherry::ura3 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5</i>	This study
<i>STE2-mCherry bar1Δ</i> (JT6677)	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2-mCherry::URA3 bar1Δ::CgLEU2</i>	Alvaro et al. (2014)
<i>STE2^{7KR}-mCherry bar1Δ</i>	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2^{7KR}-mCherry::URA3 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>	Alvaro et al. (2014)
<i>STE2^{7KR}-mCherry rod1Δ rog3Δ bar1Δ</i>	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 STE2^{7KR}-mCherry::URA3 rod1Δ::KanMX4 rog3Δ::KanMX4 bar1Δ::CgLEU2</i>	This study
<i>bni1Δ sst2Δ</i> GEV ^a	<i>MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 leu2Δ0::GEV::NatMX sst2Δ::SpHIS5 bni1Δ::KanMX4</i>	This study

^a To generate a Gal4(1-93)-estrogen receptor (ER)-VP16 chimera (designated GEV)-expressing version of the indicated yeast strain, pACT1-GEV (Veatch et al. 2009; McIsaac et al. 2011) was digested with EcoRV and introduced into the cells of interest by DNA-mediated transformation (Amberg et al. 2005), and nourseothricin-resistant colonies were selected, in which GEV (expressed under control of an *ACT1* promoter) is integrated at the *leu2Δ0* locus.

^b The immediately preceding strain was streaked onto plates containing 5-FOA, and a resulting Ura⁻ (*ura3*) derivative was selected.

amplification by the polymerase chain reaction employed Phusion DNA polymerase (New England Biolabs, Ipswich, MA), and all constructs were verified by DNA sequencing. Site-directed mutagenesis 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 (Reneke et al. 1988). In brief, cells 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 (15 μ l) of an aqueous solution (1 mg/ml) of synthetic α -factor (GeneScript, Piscataway, NJ) was aseptically spotted, and the plates were incubated at 30° for 4–5 days. In those experiments in which α -arrestin over-expression was induced, strains containing the tripartite *S. cerevisiae* Gal4-human estrogen receptor-herpes simplex virus transactivator VP16 fusion protein (Gal4-ER-VP16 or GEV) (Quintero et al. 2007) and a *URA3*-marked multi-copy (2 μ m DNA) plasmid expressing from a *GAL* promoter the α -arrestin of interest [which was fused to the C terminus of

Table 2 Plasmids used in this study

Plasmid	Genotype	Source
pEGKG	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	Yeast Deletion Collection (Open Biosystems, Inc.)
pEGKG-Rod1	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	Zhu <i>et al.</i> (2000)
pEGKG-Rod1 ^{315Aa}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{447Aa}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{641Aa}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{706Aa}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{720Aa}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{781Aa}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{447A 641Aa}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{447A 706Aa}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{3A (Rod1^{447A 641A 706A})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{4A (Rod1^{315A 447A 641A 706A})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{5A (Rod1^{315A 447A 641A 706A 720A})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{6SA (Rod1^{5315A S447A S641A S706A S720A S781A})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pGEX6P1-Rod1 ^{6SE (Rod1^{5315E S447E S641E S706E S720E S781E})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pGEX6P1-Rod1 ^{ARR (Rod1¹⁻⁴⁰²)a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pGEX6P1-Rod1 ^{TAIL (Rod1⁴⁰³⁻⁸³⁷)a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pGEX6P1-Rod1 ^{1SA ARR (Rod1^{1-402 S315A})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pGEX6P1-Rod1 ^{5SA TAIL (Rod1^{403-837 S447A S641A S706A S720A S781A})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{2SA (Rod1^{138A 807A})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{2SE (Rod1^{138E 807E})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{8SA (Rod1^{5315A S447A S641A S706A S720A S781A 138A 807A})a}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	This study
pEGKG-Rod1 ^{PANA (pJT4954)}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	Alvaro <i>et al.</i> (2014)
pEGKG-Rod1 ^{PASA (pJT4955)}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	Alvaro <i>et al.</i> (2014)
pEGKG-Rod1 ^{PPxY-less (pJT4956)}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	Alvaro <i>et al.</i> (2014)
pEGKG-Rod1 ^{V/PPxY-lessa}	<i>LDB19_{prom}</i> <i>CEN, HIS3</i>	This study
pEGKG-Rod1 ^{2A, V/PPxY-lessa}	<i>LDB19_{prom}</i> <i>CEN, HIS3</i>	This study
pEGKG-Rod1 ^{6A, V/PPxY-lessa}	<i>LDB19_{prom}</i> <i>CEN, HIS3</i>	This study
pEGKG-Rod1 ^{8A, V/PPxY-lessa}	<i>LDB19_{prom}</i> <i>CEN, HIS3</i>	This study
pEGKG-Rog3	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	Zhu <i>et al.</i> (2000)

(continued)

Table 2, continued

Plasmid	Genotype	Source
pEGKG-Rog3 ^{A400}	<i>GAL1_{prom}-GST</i> <i>2μ, URA3</i>	Alvaro <i>et al.</i> (2014)

^a Generated by site-directed mutagenesis (Green and Sambrook 2012b) with synthetic oligonucleotides containing the desired codon alterations (using the wild-type sequences in pRS426 vectors as the template). DNA from the corresponding gene was amplified from genomic DNA by PCR (Green and Sambrook 2012a) and then cloned into pEGKG.

glutathione S-transferase (GST)] were grown to midexponential phase, treated with β -estradiol (20 μ M final concentration) for 3 hr, and then plated in top agar also containing β -estradiol (final concentration 200 nM). To confirm α -arrestin overexpression, samples of the same cultures were analyzed by immunoblotting.

Immunoblotting

Equal numbers of cells from midexponential phase cultures were collected by centrifugation and stored at -80° . 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 precipitation (Volland *et al.* 1994). 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° for 15 min, resolved by SDS-PAGE, and analyzed by immunoblotting. To dephosphorylate phosphoproteins in extracts, protein precipitates were solubilized in sample buffer (80 mM Tris-HCl, pH 8.0, 8 mM EDTA, 120 mM DTT, 3.5% SDS, 0.29% glycerol, 0.08% Tris base, 0.01% Bromophenol blue), and then treated with 10 μ l of calf intestinal phosphatase (CIP) (10,000 units/ml) for 1 hr at 37° . The resulting samples were then resolved by SDS-PAGE and analyzed by immunoblotting. Proteins in SDS-PAGE gels were transferred electrophoretically to nitrocellulose sheets using a semidry transfer apparatus (Transblot SD; Bio-Rad, Inc.). After blocking with carrier protein, the filters were incubated (generally overnight at 4°) with one of the following primary antibodies: rabbit polyclonal anti-GST (Sigma), rabbit polyclonal anti-Rsp5 (gift of Allyson F. O'Donnell, Duquesne University, Pittsburgh), or rabbit polyclonal anti-Pgk1 (this laboratory) as a loading control. The resulting immune complexes were then detected by incubation with infrared dye (IRDye 680/800)-labeled goat anti-rabbit IgG secondary antibody, followed by visualization using an infrared imager (Odyssey; Li-Cor).

Purification of GST fusion proteins from *Escherichia coli*

Freshly transformed BL21(DE3) cells carrying a plasmid expressing wild-type or mutant versions of GST-Rod1^{ARR} (residues 1–403) or GST-Rod1^{TAIL} (residues 402–837) were grown to $A_{600\text{ nm}} = 0.6$, and protein expression was induced by the addition of isopropyl- β -D-thiogalactopyranoside (final concentration 0.5 mM). After aeration for 5 hr at 37° , cells were harvested and the GST fusion protein was purified by column chromatography on glutathione-agarose beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

The beads were washed three times with 500 μ l lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, 50 mM Tris-HCl, pH 7.4). Bound protein was eluted from the beads in SDS-PAGE sample buffer, resolved by SDS-PAGE (7.5% acrylamide gel), and analyzed by immunoblotting.

In vitro kinase assay

Purified Snf1 (gift of Benjamin Turk, Yale University, New Haven, CT) or purified analog-sensitive Ypk1(L424A) (gift of Alexander Muir, this laboratory) was incubated at 30° in protein kinase assay buffer (20 mM Tris-HCl, pH 7.2, 125 mM potassium acetate, 12 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT, 1% glycerol, 0.02% BSA, 25 mM β -glycerol phosphate, and 1 mM sodium orthovanadate) with 100 μ M γ -[³²P]ATP ($\sim 5 \times 10^5$ cpm/nmol) and 0.5 μ g of GST-fused substrate protein (prepared by expression in and purification from *E. coli*, as described above) with or without addition of Ypk1 inhibitor [1 μ M 1-(tert-butyl)-3-(3-methylbenzyl)-¹H-pyrazolo[3,4-d]pyrimidin-4-amine (3-MB-PP1)] (Burkard *et al.* 2007). After 30 min, reactions were terminated by addition of SDS-PAGE sample buffer containing 6% SDS followed by boiling for 5 min. Labeled proteins were resolved by SDS-PAGE and analyzed by autoradiography using a PhosphorImager (Molecular Dynamics Division, GE Healthcare).

Purification of GST fusion proteins from yeast

Rsp5 association with α -arrestins was assessed as described before (O'Donnell *et al.* 2013; Alvaro *et al.* 2014). Briefly, BJ5459 *GEV* cells carrying a plasmid vector (pEGKG) for expression of GST-Rod1 or GST-Rod1 derivatives containing a mutation(s) in its PPxY motifs (Rsp5-binding sites) were grown to midexponential phase and induced with β -estradiol (20 mM final concentration) for 3 hr. After harvesting by centrifugation, cells were washed and frozen in liquid N₂. Cell pellets were resuspended in 600 μ l co-immunoprecipitation (co-IP) buffer (100 mM NaCl, 0.2% Triton X-100, 15 nM EGTA, 50 mM Tris, pH 7.4) containing 5 mM *N*-ethylmaleimide and protease inhibitors [1 tablet of Complete protease inhibitor cocktail (Roche Applied Science) per 15 ml] and lysed at 4° by vigorous vortexing with ~ 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 4 hr at 4° . After washing two times with co-IP 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.

Fluorescence microscopy

Imaging of *Ste2*(7K-to-R)-mCherry was performed as described previously (Ballon *et al.* 2006). Cells were diluted in selective minimal medium, grown to midexponential phase, and treated with 20 μ M β -estradiol for 3 hr to induce expression of the GST-arrestin variants of interest. After collection by brief centrifugation in a microfuge, the cell population was immediately examined using an Olympus BH-2 upright fluorescence microscope (Olympus, Tokyo) equipped with a 100 \times objective, illuminated with a SOLA light engine (Lumencore, Beaverton, OR), and images were recorded with a CoolSNAP MYO CCD camera (Photometrics, Tuscon, AZ). Images were analyzed using Micro-Manager software (Edelstein *et al.* 2010) and ImageJ (National Institutes of Health). All images grouped together in any given figure were always scaled identically and always adjusted identically for brightness using Photoshop (Adobe).

Data and reagent availability

We will freely send all plasmids, strains, antibodies, and other research materials and procedures generated from this research to investigators at any and all nonprofit institutions for research purposes upon request.

Results

Snf1 phosphorylates Rod1 and inhibits its function in mating pathway down-regulation

The preferred carbon source for *S. cerevisiae* is glucose under both fermentative and nonfermentative conditions (Fraenkel 2003); however, when the supply of glucose is exhausted and oxygen is present, the cells can utilize nonfermentable carbon sources, such as lactate (Schüller 2003). Entry of lactate is mediated by *Jen1*, a lactate-specific permease (Casal *et al.* 1999). It has been demonstrated by the prior work of others that *Jen1* is endocytosed in a *Rod1*-dependent manner and that the role of *Rod1* in promoting *Jen1* internalization is blocked by phosphorylation of this α -arrestin by *Snf1* (yeast AMPK) (Shinoda and Kikuchi 2007; Becuwe *et al.* 2012), a protein kinase strongly activated under glucose-limiting conditions (Rubenstein and Schmidt 2007; Hedbacker and Carlson 2008). In this way, *Jen1* remains at the PM under conditions where uptake of lactate would be beneficial for continued growth of the cells. However, under other conditions that mimic glucose limitation and acutely activate *Snf1* (addition of the nonmetabolizable analog 2-deoxyglucose), *Rod1*-dependent endocytosis of two low-affinity glucose transporters (*Hxt1* and *Hxt3*) is stimulated (O'Donnell *et al.* 2015). Hence, it was not at all clear whether *Snf1* phosphorylation of *Rod1* has any effect, either positive or negative, on its ability to promote desensitization of mating pheromone response. Moreover, all of the sites in *Rod1* phosphorylated by *Snf1* have not been delineated previously.

Snf1 is strongly activated when cells are shifted from glucose to a medium containing even another sugar, such as

sucrose or galactose (Hedbacker and Carlson 2008). Hence, as a first means to examine the potential role of *Snf1*-mediated phosphorylation of *Rod1* in desensitization of the mating pheromone response pathway, we compared the ability of *Rod1* overexpression to promote adaptation on medium containing glucose vs. medium containing galactose. For this purpose, we used an agar diffusion bioassay that we have described before (Reneke *et al.* 1988; Alvaro *et al.* 2014). Specifically, in *MATa* cells lacking the RGS protein *Sst2*, upon exposure to pheromone, there is no way to prevent persistent receptor-initiated G-protein activation, and, hence, cells undergo a potent and sustained pheromone-induced G1 arrest (Chan and Otte 1982; Dohlman *et al.* 1996), manifest as a large clear zone in the lawn around a source of α -factor. Of course, if the receptor is efficiently removed by endocytosis, then there is no way to activate the G-protein, so cells have an opportunity to recover and resume growth, which is indicated by turbidity (“fill-in”) within the halo of initial growth inhibition. This fill-in is to be distinguished from the occasional large papillae that appear [which represent rare pheromone-resistant (*ste*) mutants that arise spontaneously at a significant frequency because a loss-of-function mutation in any gene product necessary for signal propagation, such as the MAPKKK *Ste20*, the MAPKKK *Ste11*, the MAPKK *Ste7*, or the MAPK *Fus3*, for example, will confer a growth advantage when α -factor is present]. In any event, as we observed before (Alvaro *et al.* 2014), when GST-*Rod1* overexpression was driven in a β -estradiol-induced manner in *MATa sst2 Δ* cells grown on glucose, the halo displayed a faint, but readily detectable, turbidity compared to control cells expressing GST alone, as expected (Figure 1A, top). In striking contrast, when grown on galactose, but otherwise under the same conditions, the identical cells displayed much larger halos, and no fill-in was observed when GST-*Rod1* was overexpressed (Figure 1A, bottom). These findings suggested that under conditions where *Snf1* is expected to be highly active, *Rod1* is ineffective in promoting desensitization.

As one approach to determine whether *Snf1*-mediated phosphorylation of *Rod1* itself, and not some other target, is responsible for the observed inhibition of the ability of overexpressed *Rod1* to promote adaptation on galactose medium, we sought to map and mutagenize all of the *Snf1* sites in *Rod1* and then test the ability of such variants to promote adaptation on both glucose and galactose. Based on phosphorylation of known physiological substrates, as well as synthetic peptides, both yeast *Snf1* and mammalian AMPK phosphorylate at Ser exclusively (*i.e.*, not Thr) within the context of a well-defined phospho-acceptor site consensus, Φ xR/KxxSxxx Φ (where Φ is a hydrophobic residue) (Hardie *et al.* 1998). This consensus phospho-acceptor site has been amply confirmed for yeast *Snf1* using more advanced synthetic peptide library arrays (Mok *et al.* 2010). Hence, it was relatively straightforward to scan the *Snf1* sequence and locate a total of six potential *Snf1* sites (Ser315, Ser447, Ser641, Ser706, Ser720, and Ser781) (Figure 1B; Supplemental Material, Figure S1, A and B). The most N-terminal

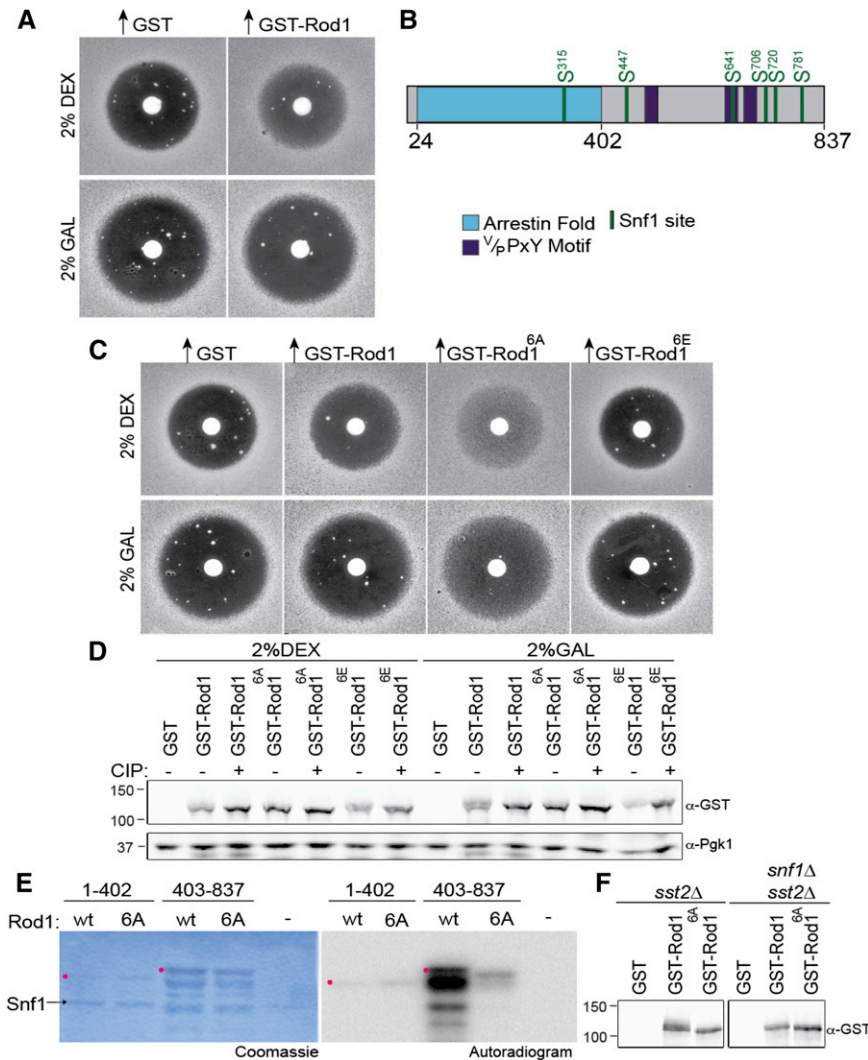


Figure 1 Snf1 phosphorylates Rod1 *in vivo* and *in vitro*. (A) *MATa sst2Δ* cells (JT6674) harboring the GEV chimera and a *URA3*-marked high-copy-number (2 μ m DNA) plasmid expressing GST-Rod1 under *GAL* promoter control were grown in minimal medium (SC-Ura) with either 2% dextrose (top) or 2% galactose (bottom) as the carbon source, induced with β -estradiol as described in *Materials and Methods*, plated in top agar on the same medium, exposed to a filter disk containing 15 μ g of α -factor, and incubated for 4 days at 30°. (B) Schematic diagram of Rod1. Arrestin fold (blue); Rsp5-binding motifs (purple); six Snf1 consensus motifs (green). (C) Same as in A, with inclusion of a nonphosphorylatable allele (Rod1^{6A}) and phospho-mimetic allele (Rod1^{6E}). (D) Samples of the cultures used in C were harvested and lysed, and the resulting extracts were divided and not treated (–) or treated (+), as indicated, with CIP, resolved by SDS-PAGE, and analyzed by immunoblotting with anti-GST or with anti-Pgk1 (loading control) antibodies. (E) GST fusions to the arrestin fold domain (residues 1–402) and the remaining C-terminal region (402–837) of either wild type (wt) or the 6A allele of Rod1 were purified from *E. coli* and incubated with [γ -³²P]ATP and purified Snf1, and the resulting products were resolved by SDS-PAGE and analyzed by autoradiography. Position of the indicated full-length GST fragment (red dot). (F) GST alone, GST-Rod1, or GST-Rod1^{6A}, as indicated, were expressed in either *SNF1*⁺ *sst2Δ* cells (left) or *snf1Δ sst2Δ* cells (right) and then analyzed by SDS-PAGE.

site is located within the arrestin fold (predicted using Phyre2.0; Kelley and Sternberg 2009), whereas the remaining five are found within or flanking the PPxY motifs in the C-terminal half of Rod1 (Figure 1B; Figure S1, A and B). Genome-wide proteomic analyses (Gnad *et al.* 2009; Soufi *et al.* 2009; Swaney *et al.* 2013) indicate that at least four of these sites (S447, S641, S706, and S720) are phosphorylated *in vivo*. Moreover, three (S447, S641, and S706) of these four sites are the most conserved in other *sensu stricto Saccharomyces* species (Figure S2A). Furthermore, one of these same sites (S447) was shown to be phosphorylated by Snf1 *in vitro* (Shinoda and Kikuchi 2007). In the same study, *rod1* (“Resistance to o-Dinitrobenzene”) loss-of-function mutations caused yeast cells to exhibit increased sensitivity to the toxic effects of 1,2-dinitrobenzene, and a Rod1(S447A) mutant conferred a modest increase in resistance to this compound (Shinoda and Kikuchi 2007). These results are consistent with a function for Rod1 in down-regulating the (unidentified) transporter(s) that mediates entry of 1,2-dinitrobenzene and a role for Snf1-mediated phosphorylation in inhibiting Rod1 function.

Hence, we used site-directed mutagenesis to convert each of these six sites alone, and in various combinations, to either a nonphosphorylatable (Ala) residue or to a phospho-mimetic (Glu) residue. We found that, when overexpressed in our *MATa sst2Δ* tester cells, Rod1(S315A S447A S641A S706A S720A S781A), henceforth abbreviated Rod1^{6A}, was much more potent than wild-type Rod1 in promoting adaptation on glucose medium, as judged by the degree of turbidity of the halo fill-in and, very importantly, was able to support readily detectable halo fill-in even on galactose medium, unlike wild-type Rod1 (Figure 1C). In marked contrast, the Rod1(S315E S447E S641E S706E S720E S781E), henceforth abbreviated Rod1^{6E}, was unable to stimulate scarcely any adaptation on either carbon source (Figure 1C). These results are fully consistent with the conclusion that *in vivo* Snf1-mediated phosphorylation is responsible for inhibiting the ability of Rod1 to promote Ste2 down-regulation on galactose medium.

The observed differences in the adaptation-promoting phenotypes among wild-type Rod1, Rod1^{6A}, and Rod1^{6E} could not be attributed trivially to any dramatic differences

in the expression levels of these proteins, as judged by immunoblotting of extracts of these same cells (Figure 1D). Moreover, and as expected, using purified *Snf1* and bacterially expressed GST-Rod1, we found that the 6A mutations virtually abolished phosphorylation of this α -arrestin at its *Snf1* sites *in vitro* (Figure 1E). Furthermore, *in vivo*, compared to glucose-grown *SNF1*⁺ cells, where the mobility of wild-type Rod1 is distinctly slower than that of Rod1^{6A}, in glucose-grown cells lacking *Snf1*, the mobility of wild-type Rod1 is increased and is very similar to that of Rod1^{6A} (Figure 1F). Thus, *Snf1* is active at a physiologically relevant level even on glucose medium.

Under our standard conditions (glucose medium), three single-site mutants, Rod1(S447A), Rod1(S706A), and Rod1(S720A), displayed a slightly enhanced ability to promote adaptation, as compared to wild-type Rod1, whereas three others, Rod1(S315A), Rod1(S641A), and Rod1(S781A), did not (Figure S2B). Indeed, S447 seems to be largely responsible for the phosphorylation-dependent mobility shift of Rod1 (Figure S2C), in agreement with the findings of Shinoda and Kikuchi (2007). Combining together as few as two of the mutations that had a detectable effect led to at least an additive improvement in its adaptation-promoting ability; for example, Rod1(S447A S706A) was somewhat more effective in promoting adaptation than Rod1(S447A S641A) (Figure S2B). Most strikingly, however, as the number of sites mutated was increased from three, to four, to five, to all six, the adaptation-promoting potency of the corresponding mutant Rod1 was incrementally increased (Figure S2B). Although the differences between the 3A, 4A, 5A, and 6A mutants are not dramatic, we continued our analysis using the most extreme mutant (Rod1^{6A}) to eliminate the contribution from all putative sites. Again, these differences could not be attributed to differences in the level of expression of these proteins (Figure S2C). Together, these data demonstrate that phosphorylation at all six *Snf1* sites occurs *in vivo* (albeit perhaps with different efficiencies at different sites) and, when phosphorylated at these sites, the ability of Rod1 to down-regulate *Ste2* is markedly impeded.

The findings discussed above indicate that *Snf1* is active at a physiologically relevant level even on glucose medium (although we cannot rule out that, in our halo bioassay, the glucose concentration may become depleted to a sufficiently low level to permit *Snf1* activation during the rather protracted time required for growth of the lawn). In this regard, however, we noted that, even when grown in liquid culture on glucose medium, and especially on galactose medium, wild-type Rod1 runs as a very diffuse band, indicative of the presence of multiple phospho-isomers (or other modifications) (Figure 1D). Treatment with phosphatase (CIP) collapsed these species to a single sharp band that comigrated with Rod1^{6A} (and the mobility of Rod1^{6A} was not significantly affected by CIP treatment) (Figure 1D). These data again indicate that wild-type Rod1 is phosphorylated at its *Snf1* sites under normal growth conditions, even on glucose medium.

We also noted that, unlike the Rod1^{6A} mutant, the Rod1^{6E} mutant displayed a mobility shift that is collapsed by CIP treatment (Figure 1D). However, it is known that, in some yeast substrates (Lee *et al.* 2012), *Snf1* phosphorylation installs a negative charge that can prime a nearby Ser for subsequent phosphorylation by casein kinase I (in *S. cerevisiae*, *Yck1*, *Yck2*, *Yck3*, and/or *Hrr25*), a protein kinase family that has a preference for phosphorylating at Ser where an Asp, Glu, or phosphorylated residue is located at position -3 (Vielhaber and Virshup 2001; Mok *et al.* 2010). We presume, therefore, that one or more of the six Glu residues present in Rod1^{6E} may create such a site(s). Moreover, at least one other yeast α -arrestin (*Rim8*) reportedly is a direct substrate for *Yck1* and *Yck2* (Herrador *et al.* 2015).

***Snf1* is not solely responsible for negative regulatory phosphorylation of Rod1**

Two observations indicated that, in cells growing on glucose, *Snf1* is likely not the sole protein kinase responsible for negative regulatory phosphorylation of Rod1. First, if *Snf1* was the major protein kinase controlling Rod1 activity on glucose, then, in a *snf1* Δ mutant, wild-type Rod1 would remain unphosphorylated and, when overexpressed, should be just as potent at promoting adaptation on glucose medium as Rod1^{6A}. However, that was clearly not the case (Figure S3).

Snf1 is the founding member of a subfamily of protein kinases, present in both yeast and mammalian cells (Alessi *et al.* 2006; Rubenstein and Schmidt 2007), that includes closely related enzymes called AMPK-like protein kinases (AMPKs). In *S. cerevisiae*, the AMPKs are the paralogous sets *Kin1* and *Kin2*, *Frk1* and *Kin4*, and *Hsl1*, *Gin4*, and *Kcc4*. We reasoned that, if any one AMPK was primarily responsible for phosphorylation of Rod1 at its *Snf1* sites when cells are grown on glucose medium that, in a loss-of-function mutant of that kinase, overexpressed wild-type Rod1 would be as potent at stimulating desensitization as Rod1^{6A}. However, in every case, Rod1^{6A} was significantly more efficacious at promoting adaptation than wild-type Rod1 in *kin1* Δ , *kin2* Δ , *frk1* Δ , *kin4* Δ , *hsl1* Δ , *gin4* Δ , and *kcc4* Δ cells (Figure S3). Of course, one or more of the AMPKs may act redundantly with each other, or with *Snf1*, with regard to Rod1 phosphorylation on glucose medium.

Three upstream kinases (*Elm1*, *Tos3*, and *Sak1*) all contribute to activation loop phosphorylation of *Snf1* (Sutherland *et al.* 2003; Elbing *et al.* 2006) and the AMPKs (Asano *et al.* 2006; Szkotnicki *et al.* 2008). Hence, as an alternative to constructing strains carrying a *snf1* Δ mutation and all possible combinations of AMPK loss-of-function mutations, we examined an *elm1* Δ *tos3* Δ *sak1* Δ triple mutant. Again, we found that Rod1^{6A} is more efficacious at promoting adaptation than wild-type Rod1 in the *elm1* Δ *tos3* Δ *sak1* Δ *sst2* Δ strain (Figure S3), although the *elm1* Δ *tos3* Δ *sak1* Δ *sst2* Δ mutant cells are rather slow-growing, making the distinctions a bit harder to discern unambiguously. Nonetheless, these findings suggested that yet another class of protein kinase might be involved in controlling Rod1 function in cells growing on glucose.

Indeed, a second observation supported the conclusion that an additional protein kinase must negatively regulate *Rod1* function on glucose medium. Specifically, despite the fact that *Rod1*^{6A} already lacks phosphorylation at all of its *Snf1* sites, its potency in promoting adaptation is lost almost completely in calcineurin (CN)-deficient cells (see later section *Calcineurin dephosphorylates the Ypk1 sites in Rod1*), indicating that phosphorylation(s) at another position(s) also needs to be removed to allow *Rod1* to function. In this regard, we noted that *Rod1* (and several other α -arrestins) were recovered in a global screen that we conducted for potential substrates of the target-of-rapamycin (TOR) complex-2 (TORC2)-activated protein kinase *Ypk1* (Muir *et al.* 2014).

Ypk1 phosphorylates Rod1 and inhibits its function in mating pathway down-regulation

It has been well established that the TORC2-*Ypk1* signaling axis regulates the sphingolipid content and other aspects of the lipid composition of the PM (Olson *et al.* 2016). Hence, it was an intriguing possibility that, through effects on the function of α -arrestins, that TORC2-*Ypk1* signaling may also regulate the protein composition of the PM. Like *Snf1*, *Ypk1* has a well-defined phospho-acceptor site motif, **RxRxxS(Φ)** (Casamayor *et al.* 1999; Mok *et al.* 2010; Muir *et al.* 2014), and *Rod1* contains two matches to this consensus: Ser138 within the arrestin fold and Ser807 near its C terminus (Figure 2A; Figure S1, A and B). Genome-wide proteomic analyses (Gnad *et al.* 2009; Swaney *et al.* 2013) indicate that both sites are phosphorylated *in vivo* and both sites are conserved in other *sensu stricto* *Saccharomyces* species (Figure S4).

As we did with the predicted *Snf1* sites, we used site-directed mutagenesis to generate *Rod1*(S138A S807A), hereafter *Rod1*^{2A}, and *Rod1*(S138E S807E), hereafter *Rod1*^{2E}, and tested their ability to promote recovery from pheromone-induced growth arrest, compared to wild-type *Rod1* and the *Snf1*-site mutant *Rod1*^{6A}, using the halo bioassay. Strikingly, *Rod1*^{2A} was significantly more potent than wild-type *Rod1* and just as potent, if not more so, than *Rod1*^{6A}, in stimulating adaptation on glucose medium (Figure 2B). Conversely, *Rod1*^{2E} promoted scarcely any desensitization, nearly comparable to the large clear halo observed for the control (GST alone) cells (Figure 2B). The dramatic difference in the phenotypes between *Rod1*^{2A} and *Rod1*^{2E} could not be attributed to any difference in their level of expression (Figure 2C). Therefore, phosphorylation of *Rod1* at its *Ypk1* sites clearly has a role in negatively regulating the function of this α -arrestin in post-pheromone response adaptation.

Unlike removal of the six *Snf1* phosphorylation sites, which largely eliminated the smear of phospho-isoforms exhibited by wild-type *Rod1* when examined by SDS-PAGE (Figure 1D and Figure 2D), removal of both *Ypk1* phosphorylation sites did not change the migration pattern markedly, and treatment with CIP collapsed the species present to a single more prominent band. Thus, these data suggest that phosphorylation occurs independently at both the *Ypk1* and *Snf1* sites *in vivo*.

In the global screen that identified *Rod1* as a candidate *Ypk1* substrate, a fragment of *Rod1* containing the C-terminal *Ypk1* site purified from bacteria was phosphorylated in a *Ypk1*-dependent manner in an *in vitro* protein kinase assay that utilized purified *Ypk1*(L424A) (*Ypk1*-as), a derivative that is sensitive to inhibition by the adenine analog 1-MB-PP1 (Muir *et al.* 2014). Using the same approach, we reproduced this result (Figure 2E). We also found that a fragment of *Rod1* containing its N-terminal *Ypk1* site was phosphorylated much less efficiently and only very weakly above the inhibitor-containing sample (Figure 2E). However, the *in vitro* assay may be misleading if the N-terminal fragment is a poor substrate simply because it lacks a high-affinity docking site for *Ypk1*. Hence, in intact *Rod1*, both its N-terminal and C-terminal *Ypk1* sites may be phosphorylated in a *Ypk1*-dependent manner *in vivo*.

If both *Snf1*- (and/or AMPKL-) and *Ypk1*-dependent phosphorylation contributes to negative regulation of the desensitization-promoting function of *Rod1*, the combination of the *Rod1*^{6A} and *Rod1*^{2A} alleles should generate a molecule the potency of which in stimulating adaptation is further enhanced. Indeed, overexpression of the resulting octuple mutant, hereafter *Rod1*^{8A}, exhibited an ability to stimulate recovery after pheromone-induced growth arrest that was reproducibly more robust than either *Rod1*^{2A} or *Rod1*^{6A} (Figure 2B and Figure 3A). These data corroborate genetically that phosphorylation by both *Ypk1* and *Snf1* (and/or a AMPKL) inhibits *Rod1* function at different sets of Ser residues. Furthermore, various global phospho-proteomics analyses (Gnad *et al.* 2009; Soufi *et al.* 2009; Swaney *et al.* 2013) indicate that other sites in *Rod1* are phosphorylated *in vivo*. Consistent with this, even the *Rod1*^{8A} derivative displays a small, but detectable, trail of slower mobility isoforms that are removed upon CIP treatment (Figure 2D); nonetheless, in the *Rod1*^{8A} mutant, the majority of the phosphorylations responsible for the mobility shifts displayed by wild-type *Rod1* have been largely eliminated.

Calcineurin dephosphorylates the Ypk1 sites in Rod1

We demonstrated before (Alvaro *et al.* 2014) that CN-mediated dephosphorylation of *Rod1* is required for its function in desensitization of mating pheromone response. Specifically, overexpression of *Rod1* in wild-type cells promotes adaptation, whereas *Rod1* overexpression in cells lacking either the paralogous CN catalytic subunits (*cna1* Δ *cna2* Δ) or their shared Ca²⁺-binding regulatory subunit (*cnb1* Δ) fails to display any detectable recovery (Figure 3A) and, based on electrophoretic mobility smearing, *Rod1* clearly remains more heavily phosphorylated in cells lacking CN than in wild-type cells (Figure 3B), as we showed before (Alvaro *et al.* 2014). Remarkably, the *Rod1*^{2A} mutant was able to promote faint, but detectable, halo fill-in in cells lacking CN, whereas *Rod1*^{6A} was barely effective at promoting adaptation in CN-deficient cells (Figure 3A), even though *Rod1*^{2A} remained more heavily phosphorylated overall than *Rod1*^{6A} in cells lacking CN (Figure 3B). More striking still, the *Rod1*^{8A} mutant was substantially more

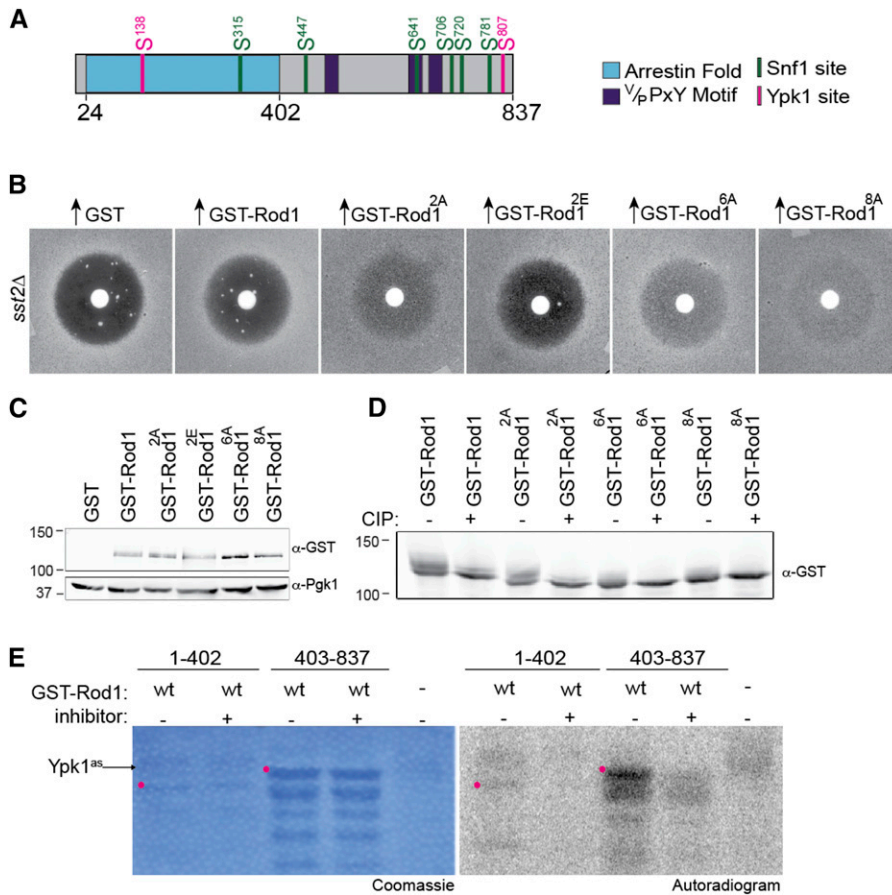


Figure 2 Ypk1 phosphorylates Rod1 *in vivo* and *in vitro*. (A) Schematic diagram of Rod1. Arrestin fold (blue); Rsp5-binding motifs (purple); six Snf1 consensus motifs (green); two Ypk1 consensus motifs (pink). (B) The adaptation-promoting capacity of the indicated alleles of Rod1 was assessed as in Figure 1A. 2A, Rod1(S138A S807A); 2E, Rod1(S138E S807E); 6A, Rod1(S315A S447A S641A S706A S720A S781A); 8A, Rod1(S138A S315A S447A S641A S706A S720A S781A S807A). (C) Expression of the Rod1 variants shown in B was assessed by harvesting the indicated cultures just prior to plating, preparing whole-cell extracts, resolving samples of those lysates by SDS-PAGE (7.5% gel), and analyzing immunoblots of the resulting gels with anti-GST or anti-Pgk1 (loading control) antibodies. (D) Phosphorylation status of the Rod1 variants shown in B was assessed as described in Figure 1D, except that the SDS-PAGE separation was performed on a 5% gel to exaggerate band shifts. (E) *In vitro* phosphorylation assay, conducted as in Figure 1E, except that purified Ypk1-as was the protein kinase added, not Snf1, in the absence (–) and presence (+) of the Ypk1-as-specific inhibitor 3-MB-PP1. Position of the indicated full-length GST fragment (red dot).

potent at promoting adaptation in CN-deficient cells than either Rod1^{2A} or Rod1^{6A} (Figure 3A). These findings suggest that CN is responsible for dephosphorylation of both the Ypk1 and Snf1 sites in Rod1, but that CN action at the former is somewhat more important to alleviate Rod1 inhibition than dephosphorylation at the latter.

As assessed by electrophoretic mobility, the sites removed from Rod1^{8A} bypass the need for CN-mediated dephosphorylation (Figure 3B). However, as efficacious as Rod1^{8A} is in promoting recovery in CN-deficient cells, Rod1^{8A} overexpression is even more potent in promoting adaptation in wild-type cells, where other cellular phosphatases can act in conjunction with CN (Figure 3A). This finding indicates that, even though the Ypk1 and Snf1 sites are clearly major points of control, Rod1^{8A} is subject to additional (albeit more minor) negative regulatory phosphorylation, consistent with the fact that, in wild-type cells, Rod1^{8A} displays a small but detectable trail of slower mobility isoforms that are removed upon CIP treatment (Figure 2D).

In any event, we have clearly pinpointed at least eight sites that are controlled by specific dephosphorylation by CN. In this regard, it has been demonstrated that all *bona fide* CN substrates possess a conserved motif (PxIxIT and variants thereof), usually accompanied by another conserved motif (ΦLxVP and variants thereof) that can be situated up to 200 or more residues away, which serve, respectively, as

primary and secondary docking sites for the binding of CN to its target protein (Grigoriu *et al.* 2013). In this regard, Rod1 possesses readily discernible matches to both sequences: 545-PQIKIE-550 and 688-LLPLP-692. We demonstrated before that a corresponding Rod1^{AQAKAA} mutant in the apparent PxIxIT site is no longer able to bind CN and displays a defect in promoting adaptation (Alvaro *et al.* 2014).

Unphosphorylated Rod1 can act in an Rsp5-independent manner

The HECT domain E3 Rsp5 and its orthologs bind via their multiple WW folds to PPxY motifs (or variants thereof) in α-arrestins (Qi *et al.* 2014a). Rsp5 possesses three WW domains (Watanabe *et al.* 2015) and Rod1 possesses two PPxY sites and one variant in its C-terminal half (residues indicated): PPNY (487–490), VPSY (639–642), and PPAY (656–659) (Figure 1B). We previously showed, in otherwise wild-type MATa cells growing in glucose medium, that mutants lacking either the first, the third, or both sites (Rod1^{PANA}, Rod1^{PAAA}, and Rod1^{PPxY-less}) were, unlike wild-type Rod1, incapable of promoting adaptation (Alvaro *et al.* 2014). Moreover, compared to wild-type Rod1, GST-Rod1^{PPxY-less} exhibited markedly reduced binding to Rsp5 *in vivo*, as judged by pull-down assays from cell extracts, and displayed drastically reduced *in vitro* modification by purified Rsp5 in ubiquitylation assays (Alvaro

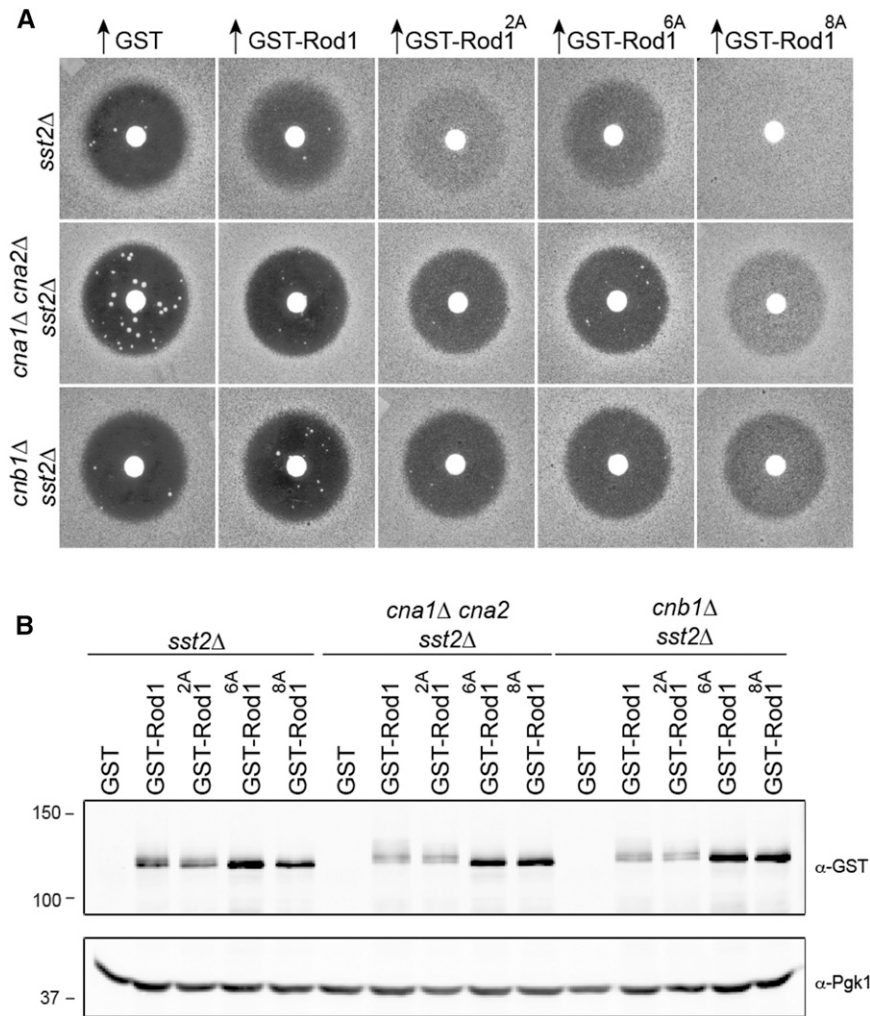


Figure 3 The requirement for calcineurin-dependent dephosphorylation of Rod1 to promote adaptation is bypassed by nonphosphorylatable Rod1 alleles. (A) The adaptation-promoting capacity of the indicated alleles of Rod1 was assessed, as in Figure 1A, in otherwise isogenic *sst2Δ* tester cells that were wild type or lacked the paralogous catalytic subunits (*cna1Δ cna2Δ*) or the small regulatory subunit (*cnb1Δ*) of phosphoprotein phosphatase 2B/calcineurin. (B) Expression of the Rod1 variants shown in A was confirmed as in Figure 2C.

et al. 2014). Therefore, we concluded that, to mediate desensitization to pheromone, Rod1 must associate with Rsp5 and deliver this E3 to its target, which other evidence indicated was the α -factor receptor Ste2.

As we demonstrated here, Rod1^{2A}, Rod1^{6A}, and Rod1^{8A} are considerably more potent in promoting recovery from pheromone-induced G1 arrest than wild-type Rod1. One possible explanation for this enhancement of function is that the lack of phosphorylation allows for higher-affinity binding of Rsp5. As one means to address this issue, we tested whether the function of Rod1^{2A}, Rod1^{6A}, or Rod1^{8A} requires intact V/PPxY motifs. Quite unexpectedly, we found that derivatives of Rod1^{2A}, Rod1^{6A}, and Rod1^{8A} in which all three motifs were mutated (PPNY → PANA, VPSY → VASA, and PPAY → PAAA), hereafter Rod1^{V/PPxY-less}, retained their ability to promote adaptation more robustly than wild-type Rod1 (Figure 4A). These properties were not due to any differences in the level of expression of these proteins (Figure 4B). Remarkably, however, the adaptation-promoting ability of Rod1^{2A}, Rod1^{6A}, or Rod1^{8A} clearly does not require intact V/PPxY motifs in these proteins, and, thus, the ability to interact with Rsp5 is not necessary for their

potent desensitization of pheromone response (Figure 4A). This finding suggests that, when unphosphorylated, Rod1 acts more like its paralog Rog3, in that it becomes able to promote adaptation in an Rsp5-independent manner, as we demonstrated for Rog3 previously (Alvaro *et al.* 2014). Indeed, we confirmed that the V/PPxY-less versions of Rod1^{2A}, Rod1^{6A}, and Rod1^{8A} all lost high-affinity binding to Rsp5 (Figure 4C).

We used the Rod1^{V/PPxY-less}, instead of the Rod1^{PPxY-less} (Alvaro *et al.* 2014) because we found that when the Rod1^{8A} allele was combined with the PANA PAAA double mutation (*i.e.*, Rod1^{PPxY-less}) it retained its recovery-promoting ability (data not shown). One possibility to explain this result was that the remaining VPSY site might be sufficient to recruit Rsp5, a similar concern we had for its paralog Rog3 (Alvaro *et al.* 2014). Indeed, using GST pull-downs, it was clear that the VPSY site contributes to Rsp5 binding to Rod1 *in vivo* (Figure S5A). To eliminate the contribution of the VPSY site, therefore, we additionally mutated it, creating Rod1^{8A V/PPxY-less}, and found that it retained its ability to robustly promote adaptation (Figure 4A). Thus, a nonphosphorylatable version of Rod1 bypasses the need for Rsp5 binding.

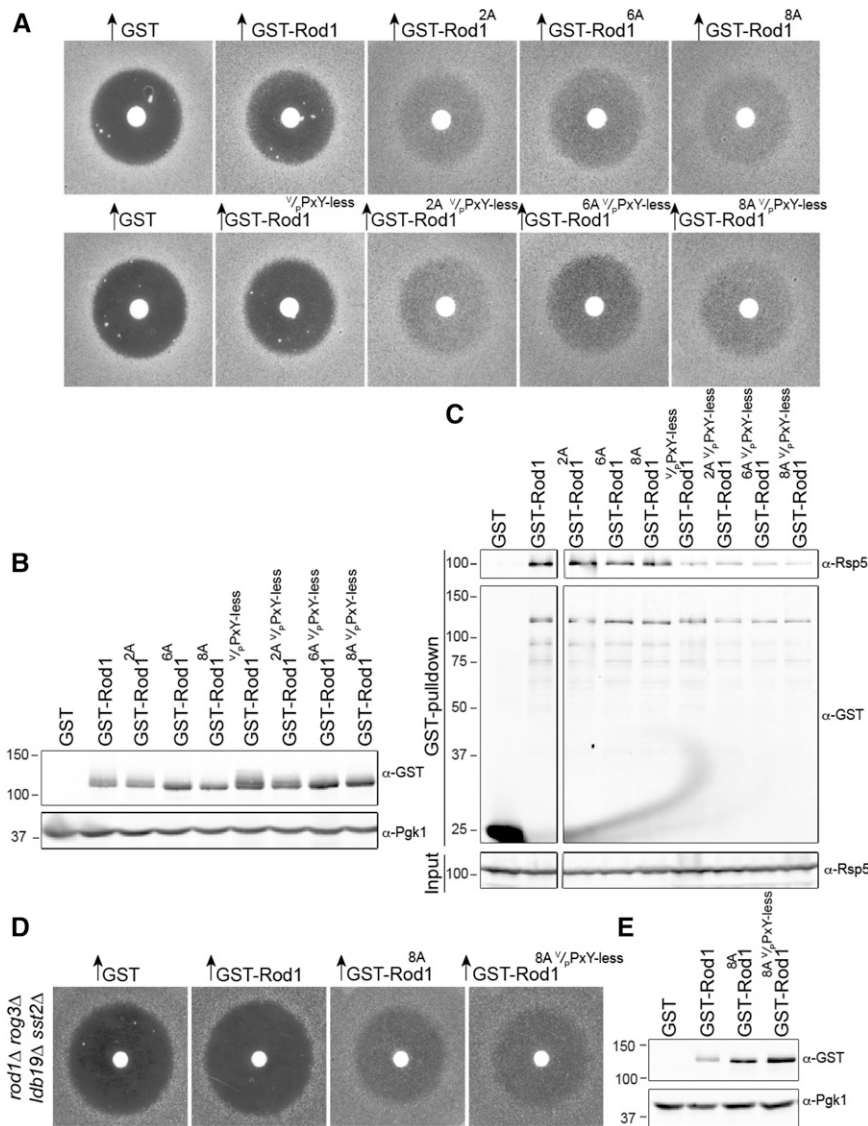


Figure 4 Hypophosphorylated Rod1 does not require Rsp5 binding to squelch mating pheromone-evoked growth arrest. (A) The adaptation-promoting capacity of the indicated 2A, 6A, and 8A alleles of Rod1 was assessed, as in Figure 1A, with or without mutation of all three Rsp5-binding motifs (V_P PxY-less). (B) Expression of the Rod1 variants shown in A was confirmed as in Figure 2C. (C) Cultures of a GEV-carrying derivative of the protease-deficient strain BJ5459 expressing the indicated Rod1 mutant were grown to midexponential phase. Protein expression was induced with β -estradiol and, after 3 hr, the cells were harvested by centrifugation and ruptured by vigorous vortex mixed with glass beads, and the GST-fusion proteins in the resulting extracts were captured by binding to glutathione-agarose beads. After washing, bead-bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. (D) The adaptation-promoting capacity of the indicated Rod1 alleles was assessed, as in Figure 1A, in mutant cells lacking endogenous Rod1, Rog3 and Ldb19. (E) Expression of the Rod1 variants shown in A was confirmed as in Figure 2C.

Interestingly, when we compared wild-type Rod1, Rod1^{PPxY-less}, and Rod1^{V/PPxY-less} (with none of the eight serines mutated), we found that Rod1^{V/PPxY-less} causes a degree of adaptation similar to that of wild-type Rod1, unlike Rod1^{PPxY-less} (Figure S5B). However, we attribute this difference to the fact that Rod1^{V/PPxY-less} was expressed at a higher level than either wild-type Rod1 or Rod1^{PPxY-less} (Figure S5C).

Another possibility to explain the fact that the V/PPxY-less versions of Rod1^{2A}, Rod1^{6A}, and Rod1^{8A} retain their potency in promoting adaptation is that these α -arrestin mutants are still able to recruit Rsp5 by forming homo-oligomers with endogenous Rod1, or hetero-oligomers with its paralog Rog3/Art7 or with the more distantly related α -arrestin Ldb19/Art1, both of which we previously showed contribute to Ste2 down-regulation (Alvaro *et al.* 2014). If so, then the partner α -arrestin could still bind Rsp5 and thereby deliver this E3 *in trans* to its target. However, even in triple-mutant cells (*rod1Δ rog3Δ ldb19Δ*) lacking all three of these other

potential partners, Rod1^{8A} and Rod1^{8A V/PPxY-less} were equally efficacious in promoting recovery from pheromone-induced growth arrest (Figure 4D) and were expressed at an equivalent level (Figure 4E). Thus, the Rod1^{8A V/PPxY-less} mutant is able to act alone to promote adaptation without recruitment of Rsp5. Thus, Rod1 has both Rsp5-dependent and Rsp5-independent mechanisms for down-regulation of mating pathway signaling, and these different adaptation-promoting functions are clearly modulated by the state of phosphorylation of this α -arrestin.

Rod1 and Rog3 action do not require the C-terminal tail of Ste2

We demonstrated before that, in cells lacking Rod1, Rog3, and Ldb19, internalization of Ste2 from the PM is greatly impeded and that, normally, the actions of these α -arrestins contribute to Rsp5-mediated ubiquitinylation-dependent endocytosis of this GPCR (Alvaro *et al.* 2014). Indeed, prior work had demonstrated that seven Lys residues in

the C-terminal cytosolic tail of *Ste2* are sites of ubiquitinylation (Hicke *et al.* 1998; Terrell *et al.* 1998; Toshima *et al.* 2009) and are required for its clathrin-mediated endocytosis (Ballon *et al.* 2006; Dores *et al.* 2010). Likewise, truncations of *Ste2* that remove its entire 134-residue C-terminal cytosolic tail just after a stop-transfer sequence installed after its seventh transmembrane helix, such as *Ste2*(Δ 296-431), also prevent endocytosis of *Ste2* (Reneke *et al.* 1988; Ballon *et al.* 2006). Furthermore, we obtained some evidence that interactions with the C-terminal cytosolic tail of *Ste2* contribute to association of *Ldb19*, *Rod1*, and *Rog3* with this receptor (Alvaro *et al.* 2014). However, the abilities of *Rod1*^{2A V/PPxY-less}, *Rod1*^{6A V/PPxY-less}, and *Rod1*^{8A V/PPxY-less} to promote adaptation quite potently (Figure 4A) suggested that, in the absence of phosphorylation, a desensitization mechanism distinct from decoration of the tail of the receptor with ubiquitin and its recognition by the endocytosis machinery was occurring.

As one means to address this issue, we asked whether the *Rod1*^{8A V/PPxY-less} mutant was still able to potently promote recovery from pheromone-induced G1 arrest in cells where either *Ste2*(7K-to-R) or *Ste2*(Δ 296-431) was the sole source of this receptor. We have shown previously that these receptor variants are poorly internalized and localize predominantly to the PM (Ballon *et al.* 2006). Indeed, we found that *Rod1*^{8A V/PPxY-less} was able to stimulate recovery as efficiently in cells expressing *Ste2*(7K-to-R) or *Ste2*(Δ 296-431) as in cells expressing wild-type *Ste2* and to do so much more effectively than wild-type *Rod1* (Figure 5A). Similar to what we observed before in cells expressing wild-type *Ste2* (Alvaro *et al.* 2014), both *Rog3* and a *Rog3* truncation mutant (Δ 400) that removes all three of its V/PPxY motifs also effectively promoted recovery in cells expressing *Ste2*(7K-to-R) or *Ste2*(Δ 296-431) as the sole source of this receptor (Figure 5A). Although there were some differences in the level of expression of these proteins that may contribute to their observed phenotypes (Figure S6B), these differences are clearly not sufficient to explain their relative efficacy in promoting adaptation. Specifically, despite the level of *Rod1*^{8A V/PPxY-less} being much lower than that of *Rog3* ^{Δ 400} (Figure S6B), they both promote robust adaptation to the point where the halo of initial growth has become obscured nearly completely.

Furthermore, overexpression of these four α -arrestin variants had no effect on the PM localization of *Ste2*(7K-to-R)-mCherry (Figure 5B), indicating that the adaptation-promoting potency of these α -arrestin variants was not due to greater efficacy in driving receptor internalization. Moreover, as judged by the halo bioassay, these α -arrestin variants promoted the same degree of adaptation when the sole source of the receptor was *Ste2*(7K-to-R)-mCherry (Figure S6A) as when it was either wild-type *Ste2* or *Ste2*(7K-to-R) (Figure 5A), confirming that the mCherry tag had no interfering effect. Collectively, these data indicate that both non-phosphorylatable *Rod1* and *Rog3* are able to promote desensitization of the mating pheromone response pathway via a mechanism independent of *Rsp5*-dependent ubiquitin-mediated receptor internalization.

A prediction of the conclusion that both *Rod1* and *Rog3* act to promote adaptation via both *Rsp5*-dependent and *Rsp5*-independent mechanisms is that loss of *Rod1* and *Rog3* function in cells expressing *Ste2*(7K-to-R) as the sole source of this receptor should display an increase in sensitivity to α -factor-induced growth arrest, compared to either *rod1 Δ rog3 Δ* cells or *Ste2*(7K-to-R) cells. Indeed, as judged by the halo bioassay, we observed an additive effect of combining a *rod1 Δ rog3 Δ* double mutation with the *Ste2*(7K-to-R) mutation (Figure 5C) that was both reproducible and statistically significant (Figure 5D).

The fact that, in the absence of its phosphorylation, *Rod1* can still promote adaptation independently of *Rsp5*-mediated receptor ubiquitinylation is consistent with recent evidence that α -arrestins can contribute to cargo recognition by both clathrin-dependent and clathrin-independent mechanisms (Prosser *et al.* 2015). However, in cells lacking a component (the formin *Bni1*) required for the clathrin-independent route (Prosser *et al.* 2011, 2015), derivatives of *Rod1* that are largely unphosphorylated and unable to associate with *Rsp5*, as well as *Rog3* and a derivative that is unable to associate with *Rsp5*, still promote efficient adaptation (Figure 5E), indicating a third means by which this α -arrestin is able to promote desensitization of the pheromone-response pathway.

Discussion

Because endocytosis of many integral PM proteins in yeast is regulated by one or more of its 14 identified α -arrestins (Lin *et al.* 2008; Nikko *et al.* 2008; Becuwe *et al.* 2012; O'Donnell *et al.* 2010, 2015), including the GPCRs *Ste2* (Alvaro *et al.* 2014) and *Ste3* (Prosser *et al.* 2015), a current question in the field is how, when, and where any given α -arrestin is recruited to a particular target. Recent studies demonstrate that phosphorylation of an α -arrestin either inhibits its ability to stimulate internalization of its target (Shinoda and Kikuchi 2007; Lin *et al.* 2008; Nikko *et al.* 2008; MacGurn *et al.* 2011; Becuwe *et al.* 2012; Merhi and Andre 2012; O'Donnell *et al.* 2013) or causes the α -arrestin to function in a different way (Crapeau *et al.* 2014; O'Donnell *et al.* 2015).

As we demonstrate here, phosphorylation of *Rod1* has a profound effect in blocking the ability of this α -arrestin to promote adaptation in the mating pheromone response pathway, where its apparent target is the α -factor receptor *Ste2* (Alvaro *et al.* 2014). Phosphoproteomic analysis by others (Gnad *et al.* 2009; Soufi *et al.* 2009; Swaney *et al.* 2013) and the mutational approach described here show that under normal growth conditions *Rod1* is inhibited by phosphorylation at its predicted *Snf1* and *Ypk1* sites because preventing phosphorylation at each of the six *Snf1* sites and its two *Ypk1* sites (by mutating the corresponding Ser residues to Ala) caused *Rod1* to be more and more potent in promoting adaptation in an additive manner. Conversely, conversion of the same sites to Glu, mimicking its permanently phosphorylated state, ablated the ability of *Rod1* to stimulate adaptation. In

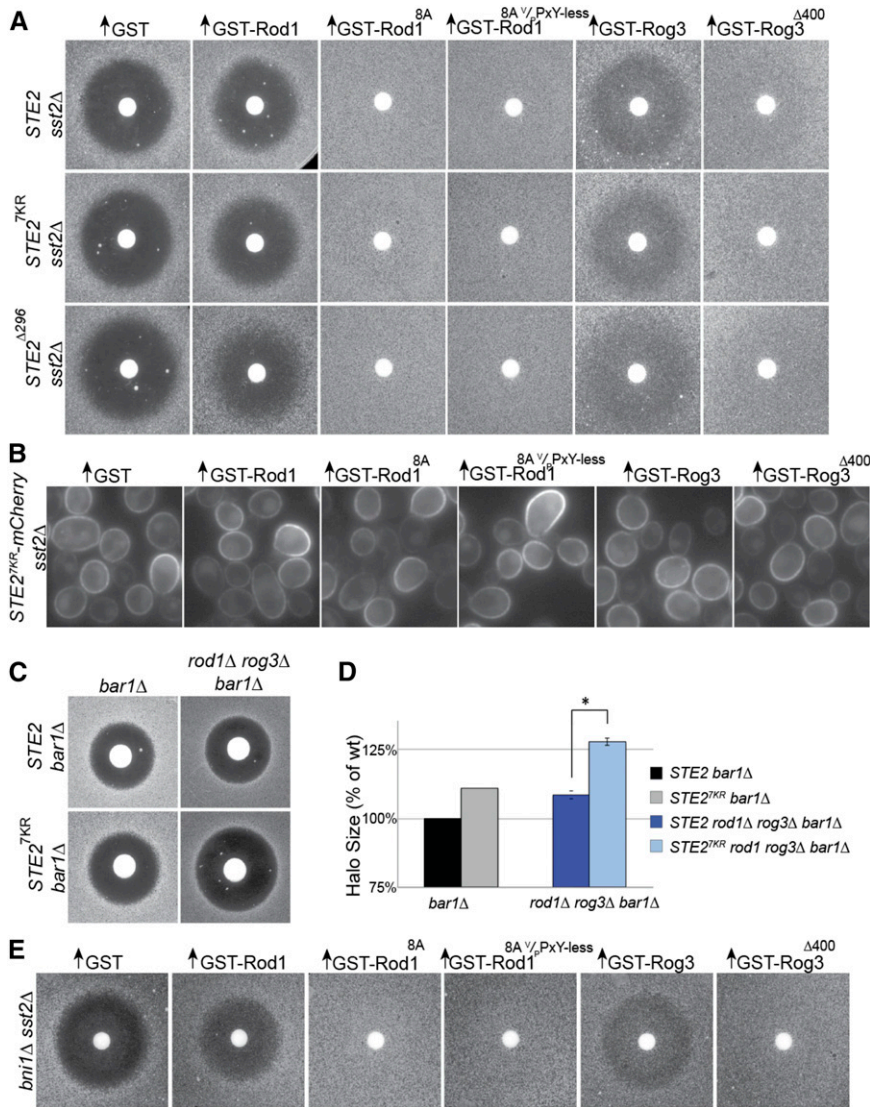


Figure 5 Hypophosphorylated Rod1 and Rog3 can stimulate adaptation independently of Ste2 ubiquitylation. (A) The adaptation-promoting capacity of the indicated alleles of Rod1 and Rog3 was assessed, as in Figure 1A, in otherwise isogenic cells expressing either wild-type Ste2, Ste2(7K-toR) (Ste2^{7KR}), or Ste2(Δ296-431) (Ste2^{Δ296}) as the sole source of this receptor. (B) Expression of the indicated GST- α -arrestins from the *GAL* promoter on *URA3*-marked 2- μ m DNA vectors was induced with 20 μ M β -estradiol for 3 hr in a *ura3* derivative of a strain expressing Ste2(7K-to-R)-mCherry from the chromosomal *STE2* locus and then examined by fluorescence microscopy. (C) Pheromone sensitivity of *MATa bar1Δ* cells, either containing or lacking endogenous Rod1 and Rog3 and expressing mCherry-tagged versions of either wild-type Ste2 or Ste2(7K-to-R), as indicated, was assessed on SC-Ura medium with sterile filter disks containing 600 ng α -factor and photographed after incubation for 2 days at 30°. (D) Quantification and statistical analysis of the change in halo diameter for independent trials ($n = 6$) of the comparative halo assays shown in C. Average halo diameter for control cells was set at 100%, and the other halo sizes of each mutant were normalized to the control. Error bars, \pm SEM; * $P < 0.001$. (E) The adaptation-promoting capacity of the indicated alleles of Rod1 and Rog3 was assessed, as in Figure 1A, in *MATa sst2Δ* tester cells lacking the formin Bni1.

this same regard, using N- and C-terminal fragments of Rod1, we found that Snf1-mediated phosphorylation of Rod1 *in vitro* occurs primarily on its C-terminal sites, and not on the one site (S315) in its arrestin fold domain. This finding suggested that, when Rod1 phosphorylation occurs *in vivo*, modification of the Snf1 sites might block Rod1 function in the main by impeding its recruitment of Rsp5 (rather than by preventing its association with Ste2). However, in pull-down experiments, Rod1^{2A}, Rod1^{6A}, and Rod1^{8A} did not bind more Rsp5 than wild-type Rod1, indicating that phosphorylation of wild-type Rod1 does not impede its association with Rsp5 *per se*. Moreover, here again the *in vitro* assay may be misleading if the N-terminal fragment is an inefficient substrate simply because it lacks a high-affinity docking site for Snf1 and/or has one-fifth the number of sites as the C-terminal fragment.

Unexpectedly, and revealingly, we found that, when phosphorylation of Rod1 is prevented on its Ypk1 sites, its Snf1 sites, or both, the corresponding Rod1 derivatives were able to promote adaptation potently, even when Rod1 was unable

to associate with the E3 Rsp5 due to mutation of all three of its V/PPxY motifs. These observations revealed that Rod1 is able to promote adaptation in an Rsp5-independent manner, similarly to what we have previously shown for its paralog Rog3 (Alvaro *et al.* 2014). Our findings thus suggest that the phosphorylation state of Rod1 dictates the mechanism by which it regulates the mating pathway.

Although phosphorylation of Rod1 by the AMPK Snf1 was shown previously to inhibit internalization of the lactate permease Jen1 (Becuwe *et al.* 2012) and stimulate internalization of the low-affinity glucose transporters Hxt1 and Hxt3 (O'Donnell *et al.* 2015), the specific phosphorylation sites in Rod1 that mediate these effects were not identified in those studies. Here, we identified six Snf1 consensus sites that are phosphorylated both *in vivo* and *in vitro*, all of which contribute to blocking the adaptation-promoting function of Rod1. When cells are grown in galactose, a condition that markedly activates Snf1 (Hardie *et al.* 1998; Hedbacker and Carlson 2008), Rod1 cannot promote adaptation; however, a Rod1^{6A}

mutant that is immune to *Snf1*-mediated phosphorylation was able to promote adaptation on galactose medium. This finding indicates that *Snf1* action inhibits the ability of *Rod1* to down-regulate the mating pathway. This phosphorylation-based mechanism makes physiological sense because it helps ensure that haploid cells will have the highest level of receptor and, hence, the greatest responsiveness to pheromone, on carbon sources other than glucose, where the capacity to mate and form diploid cells (which can sporulate when carbon is limiting) will have the greatest survival value for this organism.

We also observed that *Rod1*^{6A}, in which all the sites for *Snf1* were converted to Ala, promoted adaptation more robustly than wild-type *Rod1* even when cells are grown in glucose, a condition where *Snf1* activity is quite low. This result suggested that, on glucose (i) basal *Snf1* activity is nonetheless sufficient to inhibit *Rod1* and/or (ii) a related protein kinase of the AMPKL family is responsible for phosphorylation of these sites. Although *Snf1* displays detectable basal activity under high-glucose conditions (McCartney *et al.* 2014; O'Donnell *et al.* 2015), *Rod1*^{6A} still exhibited much more potent adaptation than wild-type *Rod1* in cells lacking *Snf1*. This result favors the latter possibility; however, deletion of no one AMPKL caused any dramatic enhancement in the adaptation-promoting ability of wild-type *Rod1*. Hence, it is possible that there is some degree of redundancy among the AMPKLs to phosphorylate *Rod1* at its *Snf1* sites. To address this possibility, we examined cells that lack the three upstream protein kinases (*Elm1*, *Sak1*, and *Tos3*) that are known activators of *Snf1* and the other AMPKLs, which again did not cause any significant enhancement in the adaptation-promoting ability of wild-type *Rod1*. However, several of the AMPKLs are known to possess significant activity even in the absence of their T-loop phosphorylation (Asano *et al.* 2006; Szkotnicki *et al.* 2008; B. Gullbrand and J. Thorner, unpublished data); hence, it is still possible that certain AMPKLs redundantly phosphorylate *Rod1* at its *Snf1* sites when cells are grown in glucose.

In agreement with a global screen that identified *Rod1* (as well as two other α -arrestins, *Rog3* and *Aly2*) as potential substrates for protein kinase *Ypk1* (Muir *et al.* 2014), we also pinpointed two sites in *Rod1* that are indeed phosphorylated by *Ypk1* both *in vivo* and *in vitro* and showed that phosphorylation at these sites is also strikingly inhibitory to the adaptation-promoting function of *Rod1*. Optimal activity of *Ypk1* requires its phosphorylation by TORC2 (Roelants *et al.* 2010, 2011), and TORC2 and *Ypk1* activity are upregulated under certain stressful conditions (*e.g.*, elevated temperature) (Sun *et al.* 2012) where again enhancing the mating proficiency of haploid cells to form diploid cells with the capacity to form heat-resistant spores would offer survival value.

Although our evidence indicates that *Ypk1* and *Snf1* (and/or one or more AMPKLs) are protein kinases that make major contributions to the phospho-regulation of *Rod1*, we also found that even a *Rod1*^{8A} mutant lacking both its *Ypk1* and *Snf1* sites exhibited minor amounts of

additional isoforms that were eliminated by CIP treatment, indicating that *Rod1* function may also be controlled to at least some degree via phosphorylation by yet other protein kinases. Consistent with this possibility, in at least one global phosphoproteomic study (Swaney *et al.* 2013), phosphate was detected on Ser and/or Thr residues other than the *Ypk1* and *Snf1* sites that we mutated. For example, four such sites fit the SP/TP consensus that could make them potential CDK or MAPK targets. In this regard, it would be interesting to determine whether *Rod1* function also is controlled in a cell cycle-dependent manner and/or subject to feedback phosphorylation by *Fus3*, the MAPK specifically activated by the mating pheromone response pathway (Hao *et al.* 2007; Merlini *et al.* 2013). If *Rod1* is a target for *Fus3*, and phosphorylation by *Fus3* is also inhibitory to *Rod1*-mediated stimulation of *Ste2* internalization, such a circuit would provide a self-reinforcing mechanism for maintaining *Ste2* at the PM and thereby more sustained pheromone signaling at least in the early phase of mating pathway activation. However, at the latter stage of pheromone response, there is a marked influx of Ca²⁺ (Ohsumi and Anraku 1985; Nakajima-Shimada *et al.* 2000; Martin *et al.* 2011) sufficient to stimulate activation of CN (Withee *et al.* 1997), which we showed previously is necessary to activate the adaptation-promoting function of *Rod1* (Alvaro *et al.* 2014). As we documented here, CN activates *Rod1* function by removing the phosphorylations at both the *Ypk1* and *Snf1* sites. An open question is whether this Ca²⁺ influx also activates any calcium-activated protein kinase that may also influence *Rod1* function or other aspects of the mating process at this stage.

Perhaps the most striking aspect of our current findings is that, in the absence phosphorylation of *Rod1*, even at as few as its two *Ypk1* sites, its adaptation-promoting ability is markedly enhanced and, most surprisingly, no longer requires *Rod1* association with the E3 *Rsp5*. In our prior work, we found that *Rod1*^{PPxY-less}, which lacks two of its *Rsp5*-binding sites, is unable to stimulate recovery from pheromone-induced growth arrest (Alvaro *et al.* 2014). Here we found that, although mutating the third *Rsp5*-binding motif (VPSY) further reduced *Rsp5* binding, *Rod1*^{V/PPxY-less} displayed a slight increase in its ability to promote adaptation, suggesting that, like the absence of phosphorylation, elimination of *Rsp5* binding further promotes the *Rsp5*-independent mechanism by which *Rod1* promotes desensitization.

Collectively, our results support a model (Figure 6) in which *Rod1* has at least two distinct mechanisms for blocking the function of *Ste2* and thus preventing the mating pheromone response. First, it is incontrovertible that, in otherwise normal cells, a primary mechanism for down-regulation is that *Rod1* delivers the ubiquitin ligase *Rsp5* to the receptor, permitting its ubiquitinylation and engagement of the clathrin-dependent endocytosis machinery, followed by internalization and destruction of *Ste2* in the vacuole (Alvaro *et al.* 2014). However, our mutational studies revealed that, when hypophosphorylated, *Rod1* can potently dampen pheromone-initiated signaling in a manner that does not require its

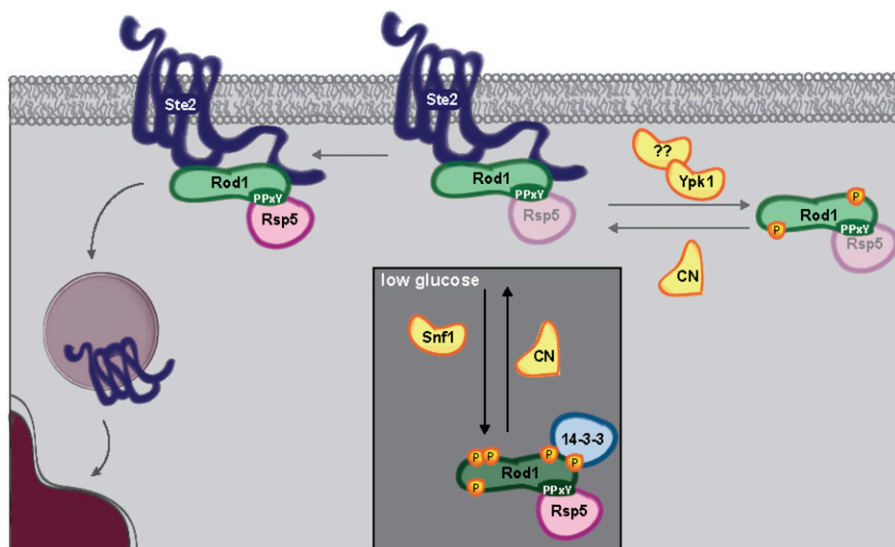


Figure 6 Phospho-regulation of Rod1 function in mating pathway desensitization. Under normal growth conditions, Rod1 is phosphorylated at multiple sites that do not prevent its productive association with Ste2. Conditions that activate the phosphoprotein phosphatase calcineurin, or that diminish the activities of the protein kinases Snf1 and Ypk1, or both, permit Rod1-receptor association, promoting the Rsp5-dependent ubiquitylation and clathrin-mediated endocytosis of Ste2. When phosphorylation of Rod1 at its Snf1 and Ypk1 sites is blocked, the only way it can be removed from the receptor is via its own Rsp5- and ubiquitin-dependent and proteasome-mediated destruction. When Rod1 cannot be phosphorylated at its Snf1 and Ypk1 sites and its V/PPxY are mutated (preventing Rsp5 recruitment), Rod1 remains bound to Ste2, blocking the ability of the receptor to stimulate its cognate G-protein and thereby potentially squelching mating pheromone-evoked growth arrest.

association with Rsp5. We propose the following explanation for this second adaptation-promoting mechanism.

In the absence of the steric and electrostatic interference imposed by both phosphorylation and Rsp5 binding, we speculate that the N-terminal arrestin fold in Rod1 is freed structurally to adopt more readily a conformation similar to that of the N-terminal arrestin fold found both in β -arrestin (Shukla *et al.* 2014) and in visual arrestin (Kang *et al.* 2015b) when bound to their target receptors. In these molecules, which lack a PPxY-containing C-terminal extension that is the hallmark of the α -arrestins, the N- and C-lobes of their arrestin folds undergo a dramatic rotation with respect to one another to engage their target receptors (rhodopsin and β 2-adrenergic receptor, respectively) (Kang *et al.* 2015a). Thereby, visual arrestin and β -arrestin hold their cognate receptors in an intimate embrace, where most of the contacts do not include interactions with the C-terminal cytosolic tails of these receptors. Importantly, this binding prevents any further signaling because it is mutually exclusive with occupancy of these receptors by their cognate G-proteins (Attramadal *et al.* 1992; Lohse *et al.* 1992; Craft *et al.* 1994). Indeed, consistent with this same kind of β -arrestin-like role for unphosphorylated Rod1, we found that Rod1^{8A V/PPxY-less} could robustly promote adaptation even in cells that express a Ste2 mutant lacking its entire C-terminal tail as the sole source of this receptor.

Because it has been shown recently that, in yeast, some α -arrestins can promote a Rho1- and formin-requiring, but clathrin-independent, mechanism for internalization of certain integral PM proteins (Prosser *et al.* 2011, 2015), we considered the possibility that absence of phosphorylation and Rsp5 binding allows Rod1^{8A V/PPxY-less} to engage this clathrin-independent route for Ste2 internalization more efficiently. However, this does not appear to be the case because Rod1^{8A V/PPxY-less}-promoted adaptation was not at all reduced in cells lacking a component

(the formin Bni1) required for the clathrin-independent internalization route.

What, then, is the normal role of α -arrestin phosphorylation? Given the fact that Rod1 action is involved in the endocytosis of quite a number of other integral PM proteins (at least Jen1, Hxt1, Hxt3, and Hxt6), and when unimpeded by phosphorylation or association with Rsp5, the arrestin fold of Rod1 appears to bind very tightly to Ste2, it is possible that a primary and physiologically relevant function for phosphorylation of Rod1 is to prevent this potential sequestration by promoting dissociation of Rod1 from Ste2 (and from its other targets). Viewed in this way, control by phosphorylation enhances the dynamic recycling of Rod1 as a means to maintain an adequate cytosolic pool so that at least some Rod1 is always available for action on each of its targets in response to the correct stimulus. In the case of Rod1 in pheromone response, Rod1 action provides a mechanism to ensure clearance of Ste2 from the surface of mating cells only in response to its CN-mediated dephosphorylation triggered by the influx of Ca²⁺ that occurs at a late stage in pheromone response.

Of course, more complicated models for how phosphorylation might control Rod1 function in the processes that promote desensitization to mating pheromone are possible. In this regard, it has been reported that phosphorylation of the α -arrestins Bul1 and Bul2 alters the way in which these adaptors bind to and regulate internalization of the general amino acid permease Gap1 (Crapeau *et al.* 2014). Thus, in the same way, it is possible that differential phosphorylation, or the lack thereof, allows Rod1 to interact with components in the mating pheromone response pathway other than Ste2 in ways that may also help to squelch signaling and promote pathway down-regulation.

GPCRs are initiators of vital signal transduction pathways in all eukaryotes, and their association with arrestins (both

α - and β -arrestins in animal cells) is important to understand the control of both signal propagation and signal dampening at the molecular level. Several of the six currently recognized α -arrestins in mammalian cells have been implicated in GPCR internalization (Nabhan *et al.* 2010; Puca *et al.* 2013; Qi *et al.* 2014b). Our work sheds new light on the roles of phospho-regulation of α -arrestins in GPCR down-regulation. Thus, *S. cerevisiae* continues to serve as a useful model to explore α -arrestin function and related mechanistic aspects of GPCR biology.

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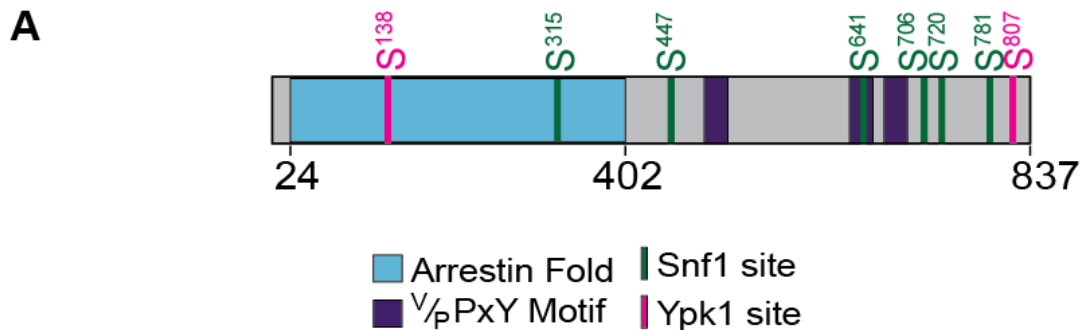
GENETICS

Supporting Information

www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.186122/-/DC1

Differential Phosphorylation Provides a Switch to Control How α -Arrestin Rod1 Down-regulates Mating Pheromone Response in *Saccharomyces cerevisiae*

Christopher G. Alvaro, Ann Aindow, and Jeremy Thorner



B

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1 MFSSSSRPSK EPLLFDIRLR NLDNDVLLIK GPPDEASSVL LSGTIVLSIT EPIQIKSLAL
61 RLFGRRLRLNI PTVLQTVHGP HKRYSKFERN IYSHFWDDFN IKSIFYQONLYD NHHNNGKITIS
121 SKSSTNLAAL PKRKRALSTA SLISSNGQTS ASKNYHTLVK GNYEFPFSAI IPGSLVESVE
181 GLPNAAVTYA LEATIERPKQ PDLICKKHLR VIRTLAIDAV ELSETVSVDN SWPEKVDYTI
241 SIPTKAI AIG SSTMINILIV PILKGLKLG P VRISLVENSQ YCGSYGGVIN QERMVAKLKL
301 KDPLKHVAQI KKRSLNEAA DEGVDTDTGE FQDKWEVRAL LNIPASLTKC SQDCRILSNI
361 KVRHKIKFTI SLLNPDGHIS ELRAALPVQL FISPFVPPVNV KTS•SDVIERTL KTFGPSYQVT
421 SQHDNSFSSK NFVDDSEEDV IFORSAS•ALQ LSSMPTIVSG STLNINSTDA EATAVADTTM
481 VTSLMVPPNY GNHVYDRVYG EVTNEDE TSA SASSSAVESQ AIHNIQONLYI SDSNNSNNPI
541 LAPNPQIKIE DDSLNNCD SR GDSVNNNSLN LVNSNLTISE NWNNSNSPSAN RYNNIINAGL
601 NSPSLTPSFA HLSRRNSYSR QTSSTSLKND LELTDLSRVP SYDKAMKSDM IGEDLPPAYP
661 EEELGVQENK KIELERPQIL HHKSTSSLLP LPGSSKSSNN LKRSS•RTHL SHSPLPRNNS
721 GSSVSLQOLA RNNTDSSFNL NLSFTSAKSS TGSRHFPFNM TTSFTSNSSS KNNSHFDKTD
781 STSDANKPRE EENYTSATHN RRSRSS•VRS NNSNSPLRQG TGSFANLMEM FTKRDRS*

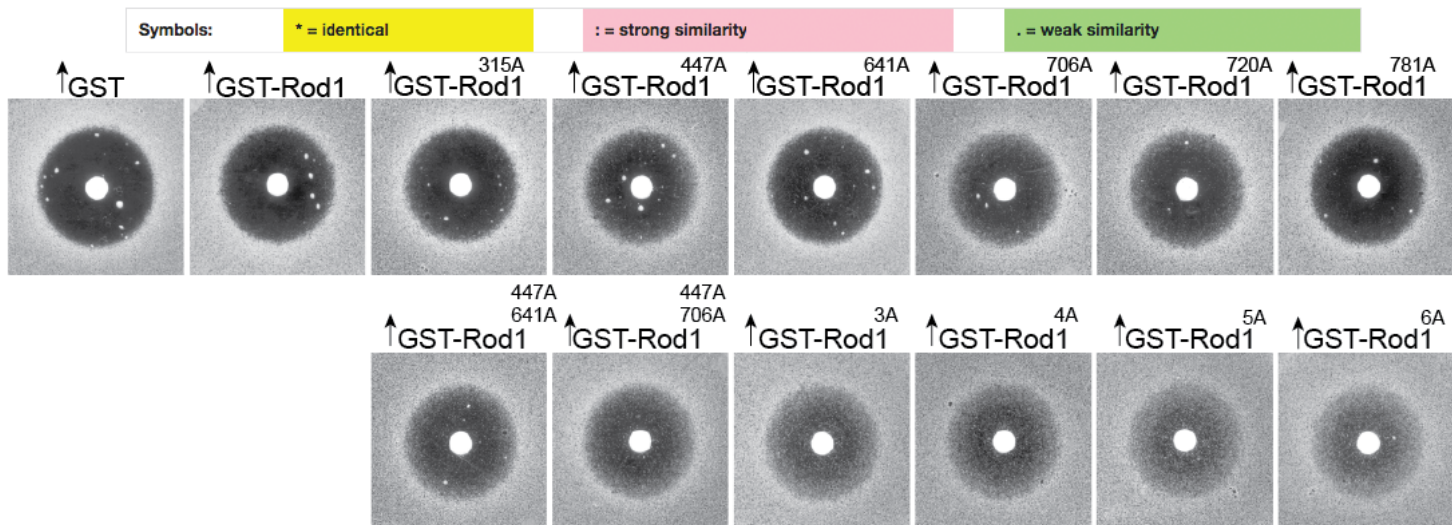
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Figure S1. Locations of phosphorylation sites for Snf1 and Ypk1 in Rod1. (A) Schematic diagram of Rod1 showing the relative positions of the six Snf1 (*green*) and two Ypk1 (*magenta*) phosphorylation sites. Arrestin fold (*blue*); V/PPxY (Rsp5-binding) motifs (*purple*). (B) Primary sequence of Rod1 with the six Snf1 consensus sites indicated by the double-underline and dotted Ser residue (*bold green*) and the two Ypk1 consensus sites indicated by the single-underline and the dotted Ser residue (*bold magenta*). Basic (R or K) residues (*bold blue*); hydrophobic residues (*bold black*); V/PPxY (Rsp5-binding) motifs (*purple*).

A

<i>S. cerevisiae</i> Rod1	298	L K L K D P L K H V A Q I K K R R S L N E A A D E G V D T S T G E F Q D K W E V R A L L N I P A S L	347
<i>S. bay</i> A pORF_10529	298	L K L K D P L K H I A Q I K K R R S L N E T N D E D F D T S T G E F Q D K W E V Q A L L N I P A S L	347
<i>S. mik</i> A pORF_20120	300	L K L R D P L K H V A Q I K K R R S L N E G A D G G I D T D I G E F Q D K W E V Q A L L N I P A S L	349
<i>S. par</i> pORF_20371	300	L K L K D P L K H V A Q M K K K R S L N E V N D E G I D I D T G E F Q D K W E V R A L L N I P A S L	349
<i>S. bay</i> B pORF_Contig606.11	298	L K L K D P L K H I A Q I K K R R S L N E T N D E D F D T S T G E F Q D K W E V Q A L L N I P A S L	347
<i>S. cas</i> pORF_Contig677.31	295	M K I K D P K K Y L S Q Y L E - - - - - N E N Y D M E N L Q F Q D K W E V D M L P L P S S L	336
<i>S. mik</i> B pORF_Contig2370.2	300	L K L R D P L K H V A Q I K K R R S L N E G A D G G I D T S T G E F Q D K W E V Q A L L N I P A S L	349
		: * : : * * * * : * : : * * * * *	
<i>S. cerevisiae</i> Rod1	398	V N V K T S D V I E R T L K T F G P S Y Q V T S Q H D N S F S S K N F V D D S E E D V I P Q R S A S	447
<i>S. bay</i> A pORF_10529	398	V N V K T S D V I E R T L K T F G P S Y Q A A T T Q N D S S F S S N K R F I D N T E E D V I P Q R S V S	447
<i>S. mik</i> A pORF_20120	400	V N V K T S D V I E R T L S T F G P S Y K V S S Q H D N S F S S N K S F T D N D T E E D V I P Q R S V S	449
<i>S. par</i> pORF_20371	400	V N V K T S D V I E R T L K T F G P S Y Q I T S Q H D N S F S S N K S F V D N D T E E D V I P Q R S V S	449
<i>S. bay</i> B pORF_Contig606.11	398	V N V K T S D V I E R T L K T F G P S Y Q A T T Q N D S S F S S N K R F I D N T E E D V I P Q R S V S	447
<i>S. cas</i> pORF_Contig677.31	387	L G V N T L D N V E A N P I K Y S H S A E T N N L V N S S I P G D - - - - - D N S R I P T T S A S	430
<i>S. mik</i> B pORF_Contig2370.2	400	V N V K T S D V I E R T L S T F G P S Y K V S S Q H D N S F S S N K S F T N D T E E D V I P Q R S V S	449
		: * : * * * * : * : : * * * * *	
<i>S. cerevisiae</i> Rod1	448	A L Q L S S M P T I V S G S T L N I N S T D A E A T A V A D T M V T S L M V P P N Y G N H V Y D R	497
<i>S. bay</i> A pORF_10529	448	E L Q L S S M P T I V S G S N L N A I N S D T E T S T T N D A M V A S L M V P P N Y G N H V Y D R	497
<i>S. mik</i> A pORF_20120	450	A L Q L S S M P T I V S G S I L N I N S T D A E A S A T A D A T M V T S L M V P P N Y G N H I Y D R	499
<i>S. par</i> pORF_20371	450	A L Q L S S M P T I V S G S T L N I N S T D A E A S A T T D A T M V T S L M V P P N Y G N H V Y D R	499
<i>S. bay</i> B pORF_Contig606.11	448	E L Q L S S M P T I V S G S N L N A I N S D T E T S T T N D A M V A S L M V P P N Y G N H V Y D R	497
<i>S. cas</i> pORF_Contig677.31	431	N M D L C I A N S E I S - - - - - S E T P M C D L M A P P N Y E K H V F D K	463
<i>S. mik</i> B pORF_Contig2370.2	450	A L Q L S S M P T I V S G S I L N I N S T D A E A S T A T D A T M V T S L M V P P N Y G N H I Y D R	499
		: : * : : : : : : : : : : : : : : : * : * * * * * : : * : : * * * * *	
<i>S. cerevisiae</i> Rod1	593	N N I I N A G L N S P S L T P S F A H L S R R N S Y S R Q T S S T S L K N D L E L T D L S R V F S Y	642
<i>S. bay</i> A pORF_10529	589	N S N T N T G L N S P S L T P S F A H L S R R N S Y S R Q T S S T S L K N D L E L T T L S R V F S Y	638
<i>S. mik</i> A pORF_20120	598	N P N I N A G L N S P S L T P S F A H L S R R N S Y S R Q T S S T S L K N E L E L T D L S R V F S Y	647
<i>S. par</i> pORF_20371	595	N N T I N A G L N S P S L T P S F A H L S R R N S Y S R Q T S S T S L K N D L E L T D L S R V F S Y	644
<i>S. bay</i> B pORF_Contig606.11	589	N S N T N T G L N S P S L T P S F A H L S R R N S Y S R Q T S S T S L K N D L E L T T L S R V F S Y	638
<i>S. cas</i> pORF_Contig677.31	553	N T I L S I G S L N E R N S I L T N P S R E S S P T L N H H S M G T R G D E L E L T D L S R V F S Y	602
<i>S. mik</i> B pORF_Contig2370.2	598	N P N I N A G L N S P S L T P S F A H L S R R N S Y S R Q T S S T S L K N D L E L T D L S R V F S Y	647
		* : : * : : : : : : : : : : : : : : * : : : : : * : : * * * * *	
<i>S. cerevisiae</i> Rod1	691	L P G S S K S S N N L K R S S R T Y L S H S P L P K N N S P S S V S L Q Q L A R N N T D S S F N L	740
<i>S. bay</i> A pORF_10529	687	F P G S S K T S N N V K S S S K G I L S H S P L P K T N N P S S V S L Q Q L T R S N T D S S F N L	736
<i>S. mik</i> A pORF_20120	696	L P G S S K S S N N V K S S S R T Y L S H S P L P K N K N P S S V S L Q Q L T R S N T D S S F N L	745
<i>S. par</i> pORF_20371	693	L P G S S K S S N S V K R S S R T Y L S H S P L P R N N S P S S I S L Q Q L T R S N T D S S F N L	742
<i>S. bay</i> B pORF_Contig606.11	687	F P G S S K T S N N V K S S S K G I L S H S P L P K T N N P S S V S L Q Q L T R S N T D S S F N L	736
<i>S. cas</i> pORF_Contig677.31	653	N - - - - - N T D G L N I T S L N R R S N N A P S S V S L D Q L L S M S S A Y L N V	689
<i>S. mik</i> B pORF_Contig2370.2	696	L P G S S K S S N N V K S S S R T Y L S H S P L P K N N S P S S V S L Q Q L T R S N T D S S F N L	745
		: : : : : : : : : : : : : : : : * : : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	
<i>S. cerevisiae</i> Rod1	741	N L S F T S A K S - S T G S R H F P F N M T P S P T S N S S S K N - - N S H F D K T I S T S D A N K	787
<i>S. bay</i> A pORF_10529	737	N L S F M A T K S - N S A S R H F P F N M T P S L A T N S S N N S Q N N L Y F G N A I S A S D T A Q	785
<i>S. mik</i> A pORF_20120	746	N L S F T S T K N - G S G S R H F P F N M T P S L A N N S G T K N - - N S F I D K T E - - - - - P K	787
<i>S. par</i> pORF_20371	743	N L S F T S T K N - G V G S R H F P F N M T P S L A S - A N S K N - - N S Y F D K T E S T S N V N K	788
<i>S. bay</i> B pORF_Contig606.11	737	N L S F M A T K S - N S A S R H F P F N M T P S L A T N S N N S Q N N L Y F G N A I S A S D T A Q	785
<i>S. cas</i> pORF_Contig677.31	690	P I G T N S S T A P S S S K F F G I N F S T G Y G S N N S E T N - - - - - - - - - - - P K	722
<i>S. mik</i> B pORF_Contig2370.2	746	N L S F T S T K N - G S G S R H F P F N M T P S L A N S G T K N - - N S F I D K T E - - - - - P K	787
		: : : : : : : * : * : * : * : * : * : : : : : : : : : : : : : : : : : * : : * : : * : : * : : *	

B



C

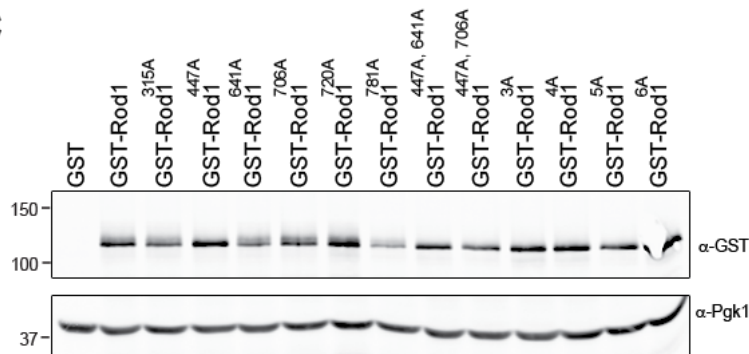


Fig. S2

Figure S2. Conservation and effect of individual Snf1 phosphorylation sites in Rod1. (A) Rod1 orthologs from the *Saccharomyces sensu stricto* species *S. cerevisiae*, *S. bayanus*, *S. mikatae*, *S. paradoxus* and *S. castellii* (Cliften *et al.*, 2003; Kellis *et al.*, 2003) were aligned using ClustalW (Thompson *et al.*, 1994). Snf1 sites in *S. cerevisiae* Rod1 (boxes); complete conservation (yellow); strong conservation (pink); weaker conservation (green). For clarity, portions of the sequence outside of those containing these sites have been omitted. (B) The adaptation-promoting capacity of the indicated single, double, triple (3A; S447A S641A S706A), quadruple (4A; S315A S447A S641A S706A), pentuple (5A; S315A S447A S641A S706A S720A) and hextuple (6A: S315A S447A S641A S706A S720A S781A) mutants was assessed as in Fig. 1A. (C) Expression of the α -arrestin variants in the cells in (B) was assessed as described in Fig. 1D.

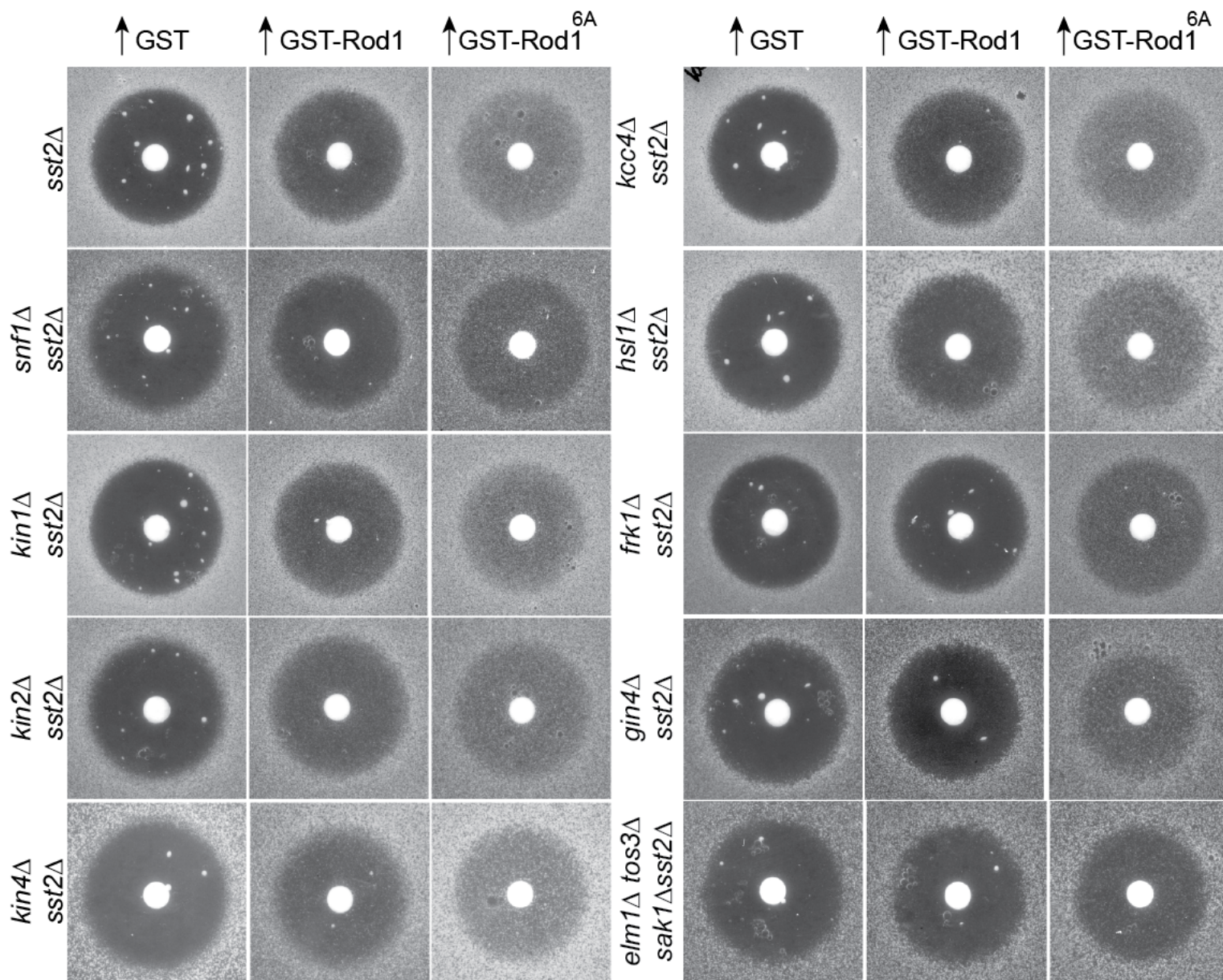


Figure S3. Contribution of AMPK-like family kinases to Rod1 regulation. The adaptation-promoting capacity of GST-Rod1 and GST-Rod1^{6A} was compared in *MATa sst2Δ* tester cells lacking either Snf1 (AMPK) or each of the other indicated members of the AMPK-like sub-family of protein kinases (AMPKs), or in a cell lacking three upstream protein kinases (Elm1, Sak1 and Tos3) known to stimulate Snf1 and other AMPKs via phosphorylation of their activation loop (*right column, bottom panels*), as in Fig. 1A.

<i>S. cerevisiae</i> Rod1	99	F N I K S Y F Q N L Y D N H N N G K I T I S S K S S T N L A A L P K R K R A L S T A S L I S S N G Q	148
<i>S. bay</i> A pORF_10529	99	F N I K N Y F Q N L Y D N H N N G K I T I S S K S S T N L A T L P K R T R A L S S T S L I S N N G Q	148
<i>S. mik</i> A pORF_20120	101	F N I K S Y F Q N L Y D N H N N G K M T I S S K S S T N L A A L P I R K R S L S T A S L I S S N G Q	150
<i>S. par</i> pORF_20371	101	F N I K S Y F Q N L Y D N H N N G K I T I S S K S S T N L A A L P K R K R A L S T A S L I S S N G Q	150
<i>S. bay</i> B pORF_Contig606.11	99	F N I K N Y F Q N L Y D N H N N G K I T I S S K S S T N L A T L P K R T R A L S S T S L I S N N G Q	148
<i>S. cas</i> pORF_Contig677.31	99	L N I K S Y I Q D L Y H N Y G K N - T S I A S K S S S N L A A M R N R G R - - S S S L V S T A S S	144
<i>S. mik</i> B pORF_Contig2370.2	101	F N I K S Y F Q N L Y D N H N N G K M T I S S K S S T N L A A L P I R K R S L S T A S L I S S N G Q	150
		: * * * . * : * : * * . * : . . . : * : * * * * : * * * : : * * : : * * : * . . .	
<i>S. cerevisiae</i> Rod1	788	P R E E N Y T S A T H N R R S R S S V R S N N S N S P L R Q G T G S F A N L M E M F T K R D R S	837
<i>S. bay</i> A pORF_10529	786	A R P E E N Y M G S V E T Q R S R S S S G R S N N S N S P S R Q K T G S F S N F M E M F T R R D R G	835
<i>S. mik</i> A pORF_20120	788	Q K A E D N S M N P T D T R R S R S S S F R S N N S N S P L R Q G T G S F A N L M E M F T K R D R S	837
<i>S. par</i> pORF_20371	789	P R E E N H T G S T H N R R S R S S S V R S N N S N S P L R Q G T G S F A N L M E M F T K R D R S	838
<i>S. bay</i> B pORF_Contig606.11	786	A R P E E N Y M G S V E T Q R S R S S S V R S N N S N S P S R Q K T G S F S N F M E M F T R R D R G	835
<i>S. cas</i> pORF_Contig677.31	723	- - - N E D L A L E I N G Q R R E S N K - - - - - - - - - - T S F A N I M G K I T R R D K K	755
<i>S. mik</i> B pORF_Contig2370.2	788	Q K A E D N S M N P T D T R R S R S S S F R S N N S N S P L R Q G T G S F A N L M E M F T K R D R S	837
		: : : . : * . * .	

Symbols:	* = identical	: = strong similarity	. = weak similarity
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Figure S4. Conservation Ypk1 phosphorylation sites in Rod1. Rod1 orthologs in *Saccharomyces sensu stricto* species aligned using ClustalW, as in Fig. S2A. Ypk1 sites in *S. cerevisiae* Rod1 (boxes); complete conservation (yellow); strong conservation (pink); weaker conservation (green). For clarity, sequences outside of those containing the sites are omitted.

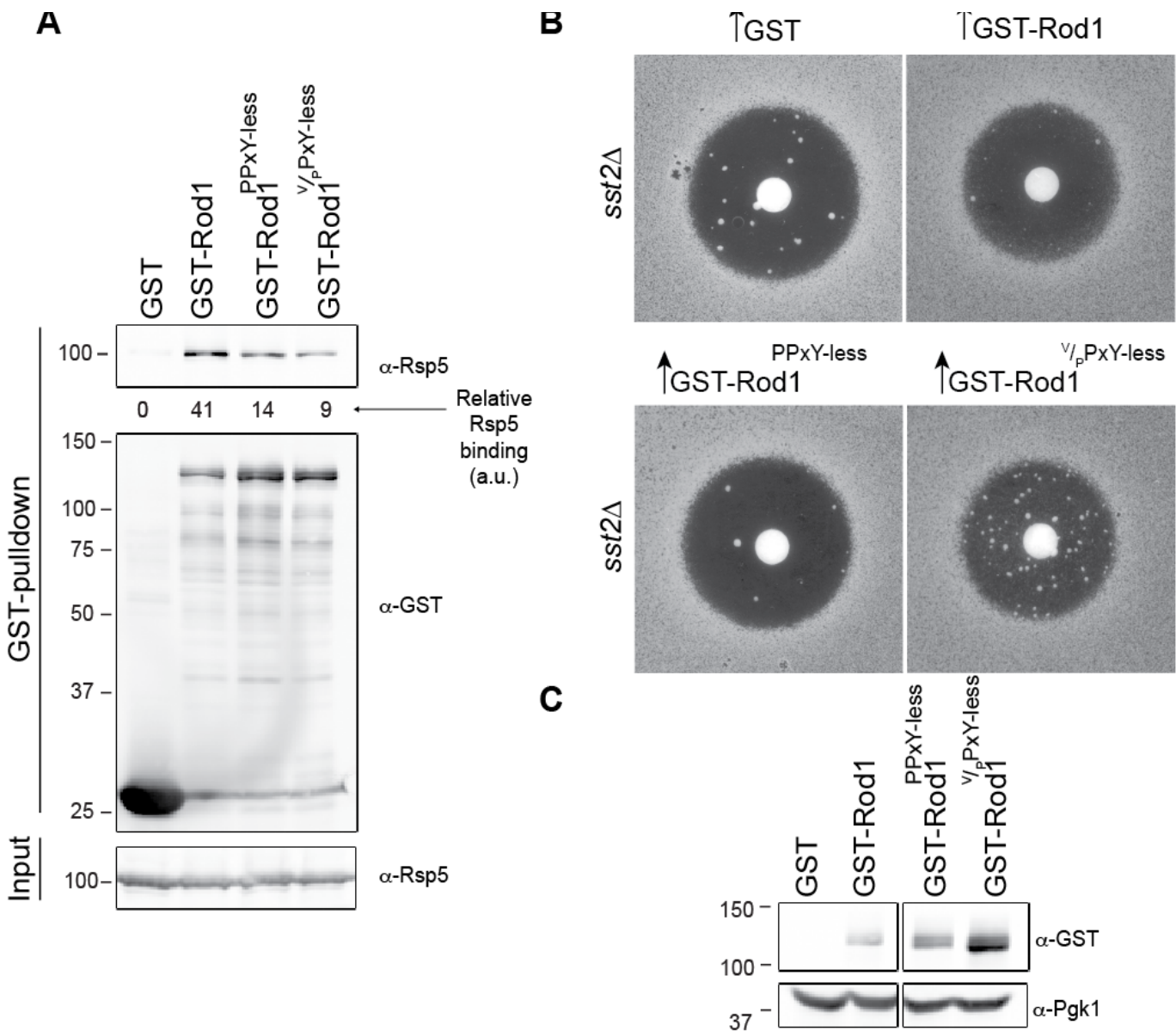


Figure S5. Removal of all three PPxY motifs slightly improves Rod1-promoted adaptation.

(A) Cultures of a GEV derivative of the protease-deficient strain BJ5459 expressing GST-Rod1 or the indicated GST-Rod1 mutant 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 mixed with glass beads. GST-fusions in the resulting extracts were captured by binding to glutathione-agarose beads. The bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. (B) The adaptation-promoting capacity of GST-Rod1 or variants lacking Rsp5-binding sites, a PANA PAAA double mutant (PPxY-less) and a VPSY PANA VASA PAAA triple mutant (V/PPxY-less). (C) Expression of the α -arrestin variants in the cells in (B) was assessed as described in Fig. 1D.

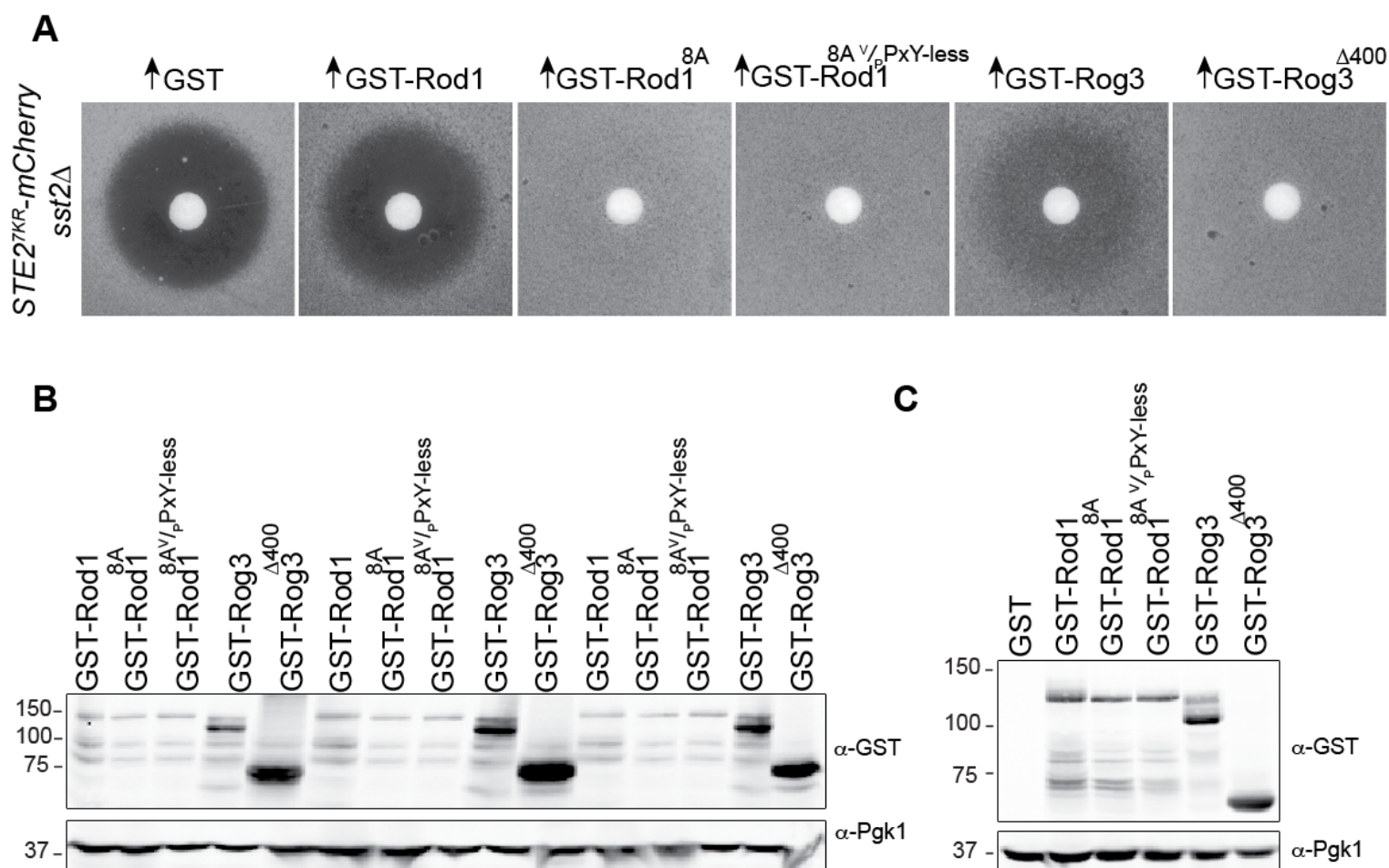


Figure S6. Overexpression of GST- α -arrestins in *STE2(7K-to-R)-mCherry sst2 Δ* and *bni1 Δ sst2 Δ* cells. (A) The adaptation-promoting capacity of the indicated alleles of Rod1 and Rog3 was assessed, as in Fig. 1A, in otherwise isogenic cells expressing Ste2(7K-toR)-mCherry (Ste2^{7KR}-mCherry) as the sole source of this receptor. (B) Expression of the GST- α -arrestin variants shown in Fig. 5A was confirmed as in Fig. 2C. (C) Expression of the GST- α -arrestin variants shown in *bni1 Δ sst2 Δ* (Fig. 5E) was confirmed as in Fig. 2C.