Regulation of *Saccharomyces cerevisiae* Mating Pheromone Response: 
G-protein-coupled Receptor Ste2 Down-modulation by Specific α-Arrestins

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Abstract

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The yeast G-protein-coupled receptor (GPCR) Ste2 is an integral plasma membrane protein that initiates response to an extracellular stimulus (the peptide mating pheromone α-factor) by mediating ligand-dependent activation of a cognate heterotrimeric G-protein. Prolonged pathway stimulation is detrimental, and feedback mechanisms have evolved that act at the receptor level to limit the duration of signaling and stimulate recovery from pheromone-induced G₁ arrest. In the research described in this dissertation, I found that three α-arrestins — Rod1/Art4, Rog3/Art7 and Ldb19/Art1 — serve as adaptors to promote the ubiquitylation-dependent internalization of Ste2 and block its ability to signal, thereby desensitizing the cells to continued stimulation. Deleting the genes encoding these three α-arrestins increases the sensitivity of a *MATα* haploid cell to mating pheromone and results in an increase in Ste2 abundance at the plasma membrane. Conversely, overexpression of either Rod1 or Rog3 enhances the rate of adaptation. To contribute to negative regulation of the mating pheromone response pathway, Ldb19 requires binding of a HECT domain class of ubiquitin ligase, Rsp5, and most likely clears misfolded Ste2 from the PM. I found that Rod1 and Rog3 contribute to clearance of the pheromone-bound state of Ste2 and negatively regulate the mating pheromone response pathway by both Rsp5-dependent and Rsp5-independent mechanisms. In addition, I identified two classes of protein kinases (Snf1/AMPK and Ypk1/SGK) that phosphorylate and inactivate Rod1. Conversely, I showed that the phosphoprotein phosphatase responsible for dephosphorylating and re-activating Rod1 is calcineurin / PP2B. Because the *S. cerevisiae* genome does not encode any β-arrestins, the findings I made and present in this dissertation are the first to show that α-arrestins alone are capable of negatively regulating a GPCR.
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CHAPTER 1: Arrestin(g) GPCRs: Regulation of G protein-coupled receptor trafficking by arrestin family proteins

To adapt to new environments, cells must sense and respond to external cues. This sensing involves receptors embedded in the plasma membrane (PM). The largest class of such cell-surface receptors in eukaryotes is the family of G-protein coupled receptors (Granier & Kobilka, 2012; Katritch et al., 2013). These proteins all possess a seven-transmembrane-segment (7-TMS) topology with the amino-terminus on the exocellular side of the PM and the carboxyl terminus facing the cytosol. For GPCRs that bind peptides and other small-molecule agonists, the cognate ligand binds to solvent-accessible contact residues displayed within the bouquet of transmembrane helices, and the receptor is bound via its cytosolic face to a signal-transducing heterotrimeric G protein (composed of a GTP-binding Gα subunit and associated Gβγ complex).

The human genome encodes nearly 1000 GPCRs (Rosenbaum et al., 2009), including ~400 specific to the olfactory system, as well as 17 Gα genes (Worzfeld et al., 2008), 5 Gβ genes and 12 Gγ genes (Khan et al., 2013). Our GPCRs are vital to our survival because they allow us to recognize and respond to stimuli ranging from light, to neurotransmitters, pruritogens, peptide hormones, odorants, and mechanical force (Fredriksson, et al. 2003; Kroeze, 2003; Katritch et al., 2011, 2013). Aside from playing roles in a wide range of physiological functions, GPCRs are also the targets of the majority of clinically-used pharmaceuticals (Shoichet & Kobilka, 2012; Zhang & Xie, 2012; Garland, 2013), making it imperative that we understand, in detail, the mechanisms underlying GPCR signal transmission and attenuation.

The mating pathway in *S. cerevisiae*

The best understood GPCR-mediated pathway in any eukaryotic cell is, arguably, the response of haploid *Saccharomyces cerevisiae* cells to their secreted peptide mating pheromones. Ste2 is the GPCR of *MATα* cells that recognizes the pheromone made by *MATα* cells (α-factor). Ste3 is the GPCR of *MATα* cells that recognizes the pheromone made by *MATα* cells (α-factor). The sequences encoding these two receptors were the second and third GPCR genes ever cloned and characterized in any eukaryote (Fuller et al., 1988; Nakayama et al., 1987). Both receptors are coupled to the same heterotrimeric G protein composed of Gpa1/Scg1 (Gα) and Ste4-Ste18 (Gβγ) and engage the same downstream components (Figure 1.1). Genetic and biochemical analysis of the events triggered by these receptors, both the gene products required for signal propagation and those involved in subsequent signal desensitization, have provided many seminal advances to our understanding of GPCR-evoked signaling and its regulation (Dohlman, 2002; Chen & Thorner, 2007; Yu et al., 2008b). A few examples of these contributions include the first demonstration that a released Gβγ complex can serve as a positive effector for signal initiation (Whiteway et al., 1989), the first identification of genes for mitogen-activated protein kinases (MAPKs), Kss1 (Courchesne et al., 1989) and Fus3 (Elion et al., 1990), for a mitogen-activated protein kinase kinase (MAPKK), Ste7 (Teague et al., 1986), and for a mitogen-activated protein kinase kinase kinase (MAPKKK), Ste11 (Rhodes et al., 1990), and discovery of the first RGS protein (Regulator of G-protein Signaling), Sst2, that terminates G-protein activation by promoting hydrolysis of the GTP bound to Gpa1 (Ballon et al., 2006;
Dohlman et al., 1996).

Upon α-factor binding to Ste2, this receptor undergoes a conformational change that allows it to act as a guanine-nucleotide exchange factor (GEF) on Gpa1 (Bender & Sprague, 1986; Edwards, Tan, & Limbird, 2000; Lefkowitz, 2004; Yao et al., 2006). GTP binding to Gpa1 causes dissociation of the Gβγ complex (Ste4-Ste18). Gβγ is thereby released to interact with downstream effectors (Whiteway et al., 1989). Thus, in yeast, the primary role of the Gα subunit is to negatively regulate the Gβγ complex (Dietzel and Kurjan, 1987; Miyajima et al., 1987). Presence of free Gβγ evokes three synergistic processes that must act in concert with each other to activate successfully the downstream MAPK cascade (Figure 1.1). These events all occur at the PM because Gβγ is firmly tethered there by virtue of S-palmitoylation (C16) and S-farnesylation (C15) of its C-terminal CaaX box, -NSNSVC\textsuperscript{C\textsubscript{16}}C\textsuperscript{C\textsubscript{15}}TLM-COOH (Hirschman and Jenness, 1999; Manahan et al., 2000).

First, freed Gβγ recruits the scaffold protein Far1, along with its interaction partner Cdc42, which is the GEF for the small (21.3 kDa) GTPase Cdc42 (Butty et al., 1998; Nern & Arkowitz, 1999). The Far1-Cdc42 complex continuously shuttles in and out of the nucleus, but is found predominantly inside the nucleus in naive cells; however, after cells are exposed to pheromone, the presence of free Gβγ allows for capture and accumulation of the Far1-Cdc42 complex at the PM (Nern & Arkowitz, 2000; Shimada, Gulli, & Peter, 2000). Cdc42, the substrate of Cdc42, is also firmly tethered at the PM by virtue of S-geranylgeranylation (C20) of its C-terminal CaaX box, -IKSSKKKC\textsuperscript{C\textsubscript{20}}AIL-COOH and a basic segment that promotes association with the headgroups of PM glycerophospholipids (Richman et al., 2004). Hence, the pheromone-induced propinquity of Cdc42 and its GEF generates a localized pool of active (GTP-bound) Cdc42.

Second, a direct target of Cdc42, the Cdc42-activated protein kinase, Ste20 – the first eukaryotic p21-activated protein kinase (PAK) identified et al., 1992; Ramer & Davis, 1993) – is also accumulated in the same vicinity because it contains a specific Gβγ-binding domain at its C-terminal end, which is also conserved in its mammalian counterparts (Leeuw et al., 1998). Thus, release of a sufficient level of free Gβγ will allow for encounter of membrane-tethered Ste20 with active membrane-anchored Cdc42, thereby leading to generation of a localized pool of the active state of this protein kinase. A primary target of Ste20 is the MAPKKK Ste11 (Wu et al., 1999; Drogen et al., 2000).

Third, freed Gβγ also recruits to the same region of the PM a second scaffold protein, Ste5, which carries as its passengers the three protein kinases of the MAPK cascade: Ste11, Ste7 and Fus3 (Elion, 2001; Good et al., 2011). By this means, Ste20 is able to encounter its substrate and trigger the phosphorylations that initiate signal propagation through the MAPK cascade (Wu et al., 1999; Drogen et al., 2000; Zalatan et al., 2012). Upon its phosphorylation, the MAPK Fus3 dissociates from Ste5 (Good et al., 2009), and is able to shuttle into the nucleus (van Drogen et al., 2001), where it activates transcription of numerous mating-specific genes (Roberts et al., 2000; Chen et al., 2010).
Figure 1.1. Summary of mating pathway in $MAT_a$ haploids. When the peptide pheromone $\alpha$-factor (green heart) binds to the GPCR Ste2 it initiates the mating pathway. The $\beta\gamma$ complex (Ste4 and Ste18), through a series of downstream targets, activates the MAPK cascade (consisting of Ste11, Ste7 and Fus3, associated with the scaffold protein Ste5). Upon phosphorylation, Fus3 shuttles into the nucleus to activate transcription of mating-specific genes and effects other processes required for mating. Gpa1, Bar1 and Sst2 are negative regulators of the mating pathway. Their regulation occurs at the level of the receptor/heterotrimeric G-protein (with Sst2 promoting regulation through its GAP activity on Gpa1).
In addition to the new gene products synthesized as the result of Fus3-mediated transcriptional induction, many of which are involved in mating-specific processes (Madhani et al., 1999; Roberts et al., 2000; Slessareva & Dohlman, 2006), such as Fus1 (a heavily O-glycosylated, PM-localized transmembrane protein required for fusion of the haploid cells) and Kar3 (a microtubule minus-end-directed kinesin required for fusion of the haploid nuclei), the actions of Fus3 also contribute to executing other steps necessary for efficient mating by phosphorylation of pre-existing proteins, including CDK1 inhibition for G1-specific growth arrest (Oehlen et al., 1998; Wittenberg & Reed, 1996), thereby synchronizing the haploid cells at the same stage of the cell cycle, and promotion of the highly polarized growth involved in conjugation tube formation (Dorer et al., 1994; Madden & Snyder, 1998), also referred to as "shmoo" formation. Because *S. cerevisiae* lack any motility mechanism, this directional chemotropic growth allows responsive haploid cells separated even by as much as 20 µm to make contact (Follette and Arkowitz, 2009).

**Negative regulation of GPCRs**
Despite the presence of a stimulus of constant intensity, cells eventually fail to propagate a GPCR-initiated signal. This physiological process, referred to as adaptation, desensitization, or down-regulation (Gainetdinov et al., 2004), is well documented in many GPCR-dependent paradigms. Desensitization represents an important biological feedback mechanism that prevents overstimulation of a GPCR-mediated signaling pathway. Because GPCRs elicit a wide array of cellular responses, aberrant GPCR signaling and dysregulation are implicated in many pathophysiological conditions, such as cancer, asthma, hypertension, neurological disorders and autoimmune diseases (West & Hanyaloglu, 2015).

Mechanisms that promote desensitization can occur at different points in a GPCR-evoked signaling pathway. However, many of these events occur at the level of the receptor itself and can be broken down, conceptually, into three major controls: (i) uncoupling of the GPCR from its cognate heterotrimeric G-protein through receptor phosphorylation (Hausdorff et al., 1989; Lohse et al., 1990a); (ii) internalization of the GPCR into internal compartments, initiated by receptor ubiquitylation (Hermans et al., 1997; Hicke & Riezman, 1996; McMahon & Boucrot, 2011; Trejo et al., 1998); and, (iii) decreasing the amount of the GPCR by (a) blocking recycling from the endosome and promoting degradation of the pre-existing receptor in the lysosome and/or (b) repressing expression of the receptor gene (Doss et al., 1981; Ferguson, 2001; Valiquette et al., 1990). The time frame for the mechanisms of desensitization summarized above range from seconds (receptor phosphorylation), to minutes (internalization), to hours (change in receptor level).

Rapid desensitization through receptor phosphorylation usually occurs on the C-terminal cytosolic tail of a GPCR and often is mediated by a class of dedicated protein kinases, termed G-protein-coupled receptor kinases (GRKs), and/or by protein kinases activated by the second messengers produced as a result of stimulation of the GPCR (Weller et al. 1975; Benovic et al., 1987; Hausdorff et al., 1989; Lohse et al., 1990a; Lefkowitz, 2013). In some cases, this phosphorylation can be a precondition for the recruitment other GPCR regulators, such as β-arrestins (discussed in detail below). Internalization of GPCRs was first suggested by observations showing that exposure to
a β2-adrenergic agonist resulted in a rapid decrease in the level of the β2-adrenergic receptor at the PM (Chuang & Costa, 1979). Since those first indirect observations, a huge amount of work has been done detailing GPCR internalization (Fisher et al., 2010), the role ubiquitin plays in this process (Xiao and Shenoy, 2011), and the fate of the receptor post-internalization (Ferguson, 2001; Tian, Kang, & Benovic, 2013; Kennedy & Marchese, 2015).

Clathrin-mediated endocytosis (CME) is the major cellular uptake mechanism for internalization of cargo proteins from the PM (Doherty & McMahon, 2009; Weinberg & Drubin, 2012). Briefly, agonist binding to a GPCR induces a conformational change in the receptor, now exposing the C-terminal tail to phosphorylation. This phosphorylation leads to adaptor protein binding, association of a ubiquitin ligase, receptor ubiquitinylation, recruitment into clathrin-coated pits (CCPs), and completion of assembly of the internalization machinery required for endocytosis. Although clathrin-independent endocytic (CIE) pathways have been identified, less is known about the mechanistic details of these forms of internalization (Kirkham & Parton, 2005; Doherty & McMahon, 2009; El-Sayed & Harashima, 2013).

**Negative regulation of the mating pathway in *S. cerevisiae***

Unregulated signaling through the mating pheromone response pathway can be deleterious and forced overstimulation can cause cell death (Zhang et al., 2006). To prevent hyperactivation and loss of cell viability, multiple negative feedback mechanisms have evolved to squelch prolonged signaling (Cyert & Thorner, 1992; Dohlman & Thorner, 2001). Several of these negative control mechanisms are exerted at the level of the pheromone receptors and their ligands. As expected for classical negative feedback mechanisms, the genes encoding these negative regulators are all pheromone-inducible. For the response of MATα cells to α-factor, these feedback controls include: upregulation of a secreted α-factor-degrading protease (Bar1) (Ciejek & Thorner, 1979; MacKay et al., 1988); upregulation of Gpa1 (Gα1), allowing for resequestration of any free Gβγ complex (Cole et al. 1990; Dohlman et al., 1993); and, upregulation of the RGS protein Sst2, which converts GTP-Gpa1 to its GDP-bound form (the state competent to recouple to Gβγ) (Dohlman et al., 1996; Ballon et al., 2006).

RGS proteins are a broad class of GPCR regulators (Dohlman & Thorner, 1997; Ross & Wilkie, 2000) that are important both for maintaining G-protein activity at a low basal level under unstimulated conditions and for adaptation after pathway activation (Burchett, 2000; Yu et al., 2008a). The tandem N-terminal DEP domains of Sst2 bind to the C-terminal tail of Ste2 (Ballon et al., 2006); this binding recruits Sst2 to the PM, permitting encounter with its substrate, GTP-bound Gpa1, which is itself firmly anchored at the PM by virtue of the N-myristoylation and S-palmitoylation (Dohlman & Thorner, 1993; Manahan et al., 2000). The C-terminal RGS domain of Sst2 stimulates hydrolysis of the GTP bound to Gpa1 (Apanovitch et al. 1998). The resulting elevated pool of GDP-Gpa1 drives reassociation with the Gβγ (Ste4-Ste18) complex, preventing further signaling through the mating pathway (Blumer & Thorner, 1990; Zhou et al., 1999). Sst2 action is essential for adaptation when cells are exposed to pheromone (Chan & Otte, 1982a, 1982b), but it is also important for preventing spurious pathway activation in the absence of pheromone (Siekhaus & Drubin, 2003). Sst2 also has been implicated in preventing receptor endocytosis in response to a low dose of pheromone.
(Venkatapurapu et al., 2015); reportedly, its binding to Ste2 post-pheromone exposure prevents phosphorylation (and presumably ubiquitinylation) of the receptor, thereby blocking its internalization. Because STE2 is a gene that is also modestly induced by pheromone (Hartig et al., 1986), the blockage of internalization purportedly increases the PM level of Ste2 when the dose of pheromone is low.

However, the claim that Sst2 modulates endocytosis is at odds with numerous other published studies which document that Ste2 (and Ste3) undergo constitutive internalization and that exposure to a physiological dose of pheromone greatly increases the rate of endocytosis (Chvatchko et al. 1986; Jenness & Spatrick, 1986; Zanolari & Riezman, 1991; Davis et al., 1993; Schandel & Jenness, 1994). Upon α-factor binding to Ste2, the ensuing conformation change exposes the C-terminal tail of the receptor permitting its phosphorylation by the PM-anchored protein kinases Yck1 and Yck2 (Panek et al., 1997; Feng & Davis, 2000). This phosphorylation serves as a prelude to efficient ubiquitinylation by the HECT-domain-containing protein:ubiquitin ligase (E3) Rsp5 (closest human ortholog is Nedd4L) (Roth & Davis, 1996; Hicke et al., 1998; Dunn & Hicke, 2001; Katzmann et al., 2002; Kamadurai et al., 2009; Stoll et al., 2011). Once ubiquitinylated, Ste2 is recruited to CCPs (Konopka et al., 1988; Reneke et al., 1988; Tan et al., 1993; Toshima et al., 2009). After clathrin-mediated endocytosis, receptor-containing vesicles are delivered to the vacuole (the yeast equivalent of the lysosome) where they are degraded (Katzmann et al., 2002; Gabriely et al., 2006). Ste2 is always trafficked to the vacuole after internalization, whereas Ste3 is recycled to the PM after internalization (much like many mammalian GPCRs) (Chen & Davis, 2002).

Rsp5 is required for both the basal and pheromone-induced internalization of Ste2 (and Ste3) (Roth & Davis, 1996; Dunn, 2001; Dunn & Hicke, 2001). In addition, the specific lysine residues ubiquitinylated by Rsp5 have been mapped in both Ste2 and Ste3 (Hicke et al., 1998; Roth & Davis, 2000; Toshima et al., 2009). In fact, mutating seven lysines to arginine in Ste2 (and three lysines to arginines in Ste3) completely prevents receptor internalization (Ballon et al., 2006). However, neither Ste2 nor Ste3 directly interact with Rsp5. Hence, one mechanistic question that remained elusive, until elucidated by the discoveries documented in this dissertation, was how Rsp5 specifically recognizes and associates with Ste2 (and Ste3) to mediate their ubiquitinylation.

Like other E3s in its class, Rsp5 has a modular structure (Figure 1.2A): an N-terminal C2 domain (which binds to the head groups of acid glycerophospholipids in the PM) (Malmberg & Falke, 2005); followed by three WW domains (protein–protein interaction modules that bind the short recognition motif, PPxY or closely related sequences, such as VPxY or LPxY) (Qi et al., 2014; Liu et al., 2015); and, a catalytic C-terminal HECT domain (which transfers a covalently attached ubiquitin to the substrate and builds onto it a K63-linked polyubiquitin chain) (Kee & Huibregtse, 2007; Kamadurai et al., 2009, 2013). Although Rsp5 is responsible for the basal and pheromone-induced ubiquitinylation of both Ste2 and Ste3, neither GPCR contains any discernible PPxY or related motif (Staub & Rotin, 1996; Dunn & Hicke, 2001; Rotin & Kumar, 2009). Other PM-localized transmembrane proteins in yeast that are also known to be internalized in a ubiquitinylation-dependent manner also lack any PPxY or related motifs that could directly bind Rsp5 (Belgareh-Touzé et al., 2008; Lin et al., 2008; Nikko & Pelham, 2009; Lauwers et al., 2010). The solution to this problem is that adaptor proteins exist that
serve as molecular matchmakers; by binding to both PM transmembrane proteins and to Rsp5, they recruit this E3 to its substrates. Such trafficking adaptors also have roles in recruiting Rsp5 to targets located in other cellular membranes, such as the Golgi compartment (O'Donnell et al., 2010; Merhi & Andre, 2012) and the vacuole (Léon et al., 2008; Li et al., 2015).

**Arrestins: a family of trafficking adaptors**

The family of trafficking adaptors described above have been dubbed arrestins and fall into three classes: \(\alpha\)-arrestins; \(\beta\)-arrestins; and, Vps26-like arrestins (Alvarez, 2008; Léon & Haguenauer-Tsapsis, 2009; Aubry & Klein, 2013). A diagnostic hallmark of all members of the arrestin family is that they share a sequence that adopts a unique three-dimensional structure, consisting of two mirror-like domains composed primarily of antiparallel \(\beta\)-sheets connected by a flexible hinge region (Figure 1.2B) (Gurevich & Gurevich, 2006). This fold is designated an arrestin fold because it was first found in bovine retinal arrestin, a member of the \(\beta\)-arrestin subfamily (Grazin et al., 1998). Although they share a similar structure, arrestin fold domains, as a group, have low sequence conservation and are, therefore, sometimes hard to identify solely by protein sequence similarity.

The first well-recognized class of the arrestin family was the \(\beta\)-arrestins (sometimes referred to as "true" arrestins). In mammalian cells, there are 4 members of the \(\beta\)-arrestin class: visual arrestin and arrestin4; both are expressed in the retina and regulate rhodopsin (Kuhn & Wilden, 1987; Craft et al., 1994), and \(\beta\)-arrestin1 and \(\beta\)-arrestin2, both initially identified as regulators of the \(\beta2\)-adrenergic receptor (Benovic et al., 1987; Lohse et. al, 1990b; Attramadal et al., 1992). \(\beta\)-arrestins preferentially bind phosphorylated (i.e., activated) forms of their target GPCR and mediate rapid desensitization by occluding the heterotrimeric G-protein from binding to and re-activation by the GPCR (Attramadal et al., 1992; Lohse et al., 1992; Craft et al., 1994). It was initially thought that \(\beta\)-arrestins play a role in GPCR internalization by binding some of the machinery for internalization (Claing et al., 2002); however, more recent work suggests that \(\beta\)-arrestins act to couple GPCR activation to other non-G-protein-dependent signaling outputs (Shukla et al., 2011; Reiter et al., 2012).

The crystal structure of light-activated rhodopsin bound to visual arrestin has been determined, providing insight into how the arrestin fold contributes to GPCR recognition (Kang et al., 2015). The arrestin binds asymmetrically to the activated GPCR. It makes multiple contacts with the C-terminal tail, the cytosolic loops, and the cytosolic ends of certain helices of the receptor. The structure also suggests that, once phosphorylated, the C-terminal tail of rhodopsin binds to arrestin and displaces auto-inhibition of arrestin by its C-terminal extension. This dis-inhibition disrupts the polar core of arrestin, allowing the N- and C-lobes of the arrestin fold to undergo a 20° rotation with respect to each other, thereby adopting a new conformation that is now compatible with making the contacts necessary for high-affinity recognition of rhodopsin recognition. This rhodopsin-arrestin complex provides the first atomic-resolution view of how an arrestin binds to a GPCR.

As mentioned above, aside from binding directly to an activated GPCR, \(\beta\)-arrestins also mediate GPCR-evoked (but non-G-protein-dependent) pathways by engaging other downstream signaling molecules, such as components of the MAPK/ERK pathway
β-arrestin1, for example, plays two roles in the response to β2-adrenergic receptor agonists. First, it helps rapidly desensitize the receptor; and, second, it binds ERK1/2, trapping the kinase in the cytoplasm and directing phosphorylation of a different subset of targets than would be affected by growth factor receptor activation of ERK1/2 (Ahn et al., 2004). Over the last decade, new scaffolding roles for β-arrestins have emerged, implicating β-arrestins in multiple signaling pathways downstream of GPCR activation (DeFea, 2011).

Another small class of arrestins is the Vps26-related arrestins. The main members of this clade are Vps26, a component of the retromer coat involved in vesicle-mediated endosome-to-Golgi retrograde protein transport (Seaman et al., 1998), and DSCR3, a MAPK-scaffold-like arrestin (Pellegrini-Calace & Tramontano, 2006). Both of these proteins share the typical arrestin structure, despite no obvious functional similarity (Alvarez, 2008; Aubry & Klein, 2013). Vps26-related arrestins have not been shown to directly regulate the ubiquitinylation and trafficking of any PM proteins.

The last class is the α-arrestins, often referred to in mammalian cells as ARRDCs (arrestin-domain containing proteins). A diagnostic hallmark of every member of the α-arrestin family is the presence of a C-terminal extension after the arrestin fold domain that contain at least one (but usually multiple) PPxY motifs (Alvarez, 2008; Lin et al., 2008; Nabhan, Pan, & Lu, 2010). PPxY motifs are not present in β-arrestins (Figure 1.2B), suggesting that it is the α-arrestins, and not the β-arrestins, that interact with E3s like Rsp5 or Nedd4L and drive the ubiquitinylation and endocytosis of GPCRs and other PM proteins. Indeed, the six identified mammalian α-arrestins, namely ARRDC1-5 and TXNIP (Alvarez, 2008; Aubry & Klein, 2013), are involved in the internalization of several different GPCRs, including β2-adrenergic receptor, β3-adrenergic receptor and others (Nabhan et al., 2010; Patwarei & Lee, 2012; Shea et al., 2012; Han et al., 2013; Kwon et al., 2013;). In addition, TXNIP has been implicated in glucose transporter endocytosis (Patwarei et al., 2009; 2011; Patwarei & Lee, 2012). The genome of S. cerevisiae does not contain any gene encoding a protein resembling a β-arrestin, but it contains at least 14 genes that encode apparent α-arrestins (Figure 1.2B). Thus, this yeast serves as a model system in which to study α-arrestin function in the absence of β-arrestin function.
Figure 1.2. Schematic of Rsp5 and α-arrestins. A. Schematic depiction of Rsp5 topology. Rsp5 contains a C2 domain, which anchors it to the plasma membrane. In addition it has three WW domains, which bind PPxY motifs in its targets. Lastly, it contains an E3-HECT ubiquitin ligase domain. B. Schematic depiction of a subset (9 out of 14) of the α-arrestins in S. cerevisiae compared to representative β-arrestin. They are grouped together with their closest paralog. In blue is the predicted Arrestin Fold domain (predicted with the program Phyre2.0). In purple are the PPxY motifs, which bind to WW domains of Rsp5.
Specific α-arrestins regulate trafficking and internalization of transmembrane proteins in *S. cerevisiae*

There are 14 currently identified α-arrestins, also referred to as Arts (arrestin-related trafficking adaptors), in *S. cerevisiae*. They all contain the signature arrestin fold, and one or more PPxY motifs or variants thereof (such as VPxY or LPxY) (Figure 1.2B). Since the initial identification and characterization of the α-arrestin class in *S. cerevisiae* (Lin et al., 2008; Nikko & Pelham, 2009; Lauwers et al., 2010), the functions of various members of this protein family have been described (Mittal & McMahon, 2009; Hatakeyama et al., 2010; Becuwe et al., 2012; Merhi & Andre, 2012; Alvaro et al., 2014; O'Donnell et al., 2015). To date, the primary role ascribed to the α-arrestins in yeast is in internalization and trafficking of nutrient permeases in response to specific stimuli. Based on these studies, an arrestin-permease interaction code has emerged (Figure 1.3), indicating both specificity and promiscuity in these interactions. Interestingly, several studies have shown that different arrestins regulate the same nutrient permease, depending on the external stimulus (Nikko & Pelham, 2009; Zhao et al., 2013; Crapeau et al., 2014; Ghaddar et al., 2014). For example, in response to high lysine, Ldb19/Art1 mediates the internalization of the lysine permease Lyp1, but in response to cycloheximide Ecm21/Art2 takes over (Nikko & Pelham, 2009). Such finding also suggest differential regulation of the α-arrestins, adding a layer of specificity to the arrestin-permease code. The α-arrestin Ldb19/Art1 seems to be involved in “quality-control” because it recruits Rsp5 to misfolded PM proteins, promoting their removal (Zhao et al., 2013). Despite the foundational work on GPCR regulation by β-arrestins, previous to the findings described in this dissertation no *S. cerevisiae* α-arrestin had been implicated in GPCR regulation.
Figure 1.3. Current arrestin-permease code in *S. cerevisiae*. A subset of the α-arrestins are shown in green. Each of the arrestins depicted has been shown to be involved in the internalization of one or more specific nutrient permeases (shown in pink). The arrestins Rim8/Art9, Art10, Bul3 and Spo23 are not depicted because a PM target for them has yet to be identified.
Three specific \(\alpha\)-arrestins regulate Ste2 in *S. cerevisiae*

In the research described in this dissertation, I found that three \(\alpha\)-arrestins, Rod1/Art4, Rog3/Art7 and Ldb19/Art1, are specifically involved in mating pathway regulation and Ste2 internalization. Deleting these three \(\alpha\)-arrestins increases the sensitivity of haploid cells to mating pheromone and results in an increase in Ste2 abundance at the plasma membrane. Conversely, overexpression of Rod1 and Rog3 enhances the rate of adaptation, as assessed by recovery and resumption of growth after pheromone-induced G1 arrest. To contribute to negative regulation of the mating pheromone response pathway, Ldb19 requires Rsp5 binding. The work describe here is consistent with a role for Ldb19 in clearing misfolded Ste2 from the PM, in agreement with its role in quality control of other classes of polytopic PM proteins (Zhao et al., 2013). As described here, Rod1 and Rog3 contribute to negative regulation of the mating pheromone response pathway by both Rsp5-dependent and Rsp5-independent mechanisms. In addition, in this work, two classes of protein kinases (Snf1/AMPK and Ypk1/SGK) that phosphorylate and inactivate Rod1 were identified. Conversely, the phosphoprotein phosphatase responsible for dephosphorylating and re-activating Rod1 (PP2B/calcineurin) was also identified. The findings presented in this dissertation are the first to show that \(\alpha\)-arrestins are capable of negatively regulating GPCRs in *S. cerevisiae* and were obtained independently and contemporaneously with studies documenting the role of \(\alpha\)-arrestins/ARRDCs in GPCR down-regulation in animal cells.
CHAPTER 2: Specific α-arrestins negatively regulate Saccharomyces cerevisiae pheromone response by down-modulating the G-protein coupled receptor Ste2

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ABSTRACT
G-protein-coupled receptors (GPCRs) are integral membrane proteins that initiate responses to extracellular stimuli by mediating ligand-dependent activation of cognate heterotrimeric G-proteins. In yeast, occupancy of GPCR Ste2 by peptide pheromone α-factor initiates signaling by releasing a stimulatory Gβγ complex (Ste4-Ste18) from its inhibitory Gα subunit (Gpa1). Prolonged pathway stimulation is detrimental, and feedback mechanisms have evolved that act at the receptor level to limit the duration of signaling and stimulate recovery from pheromone-induced G1 arrest, including up-regulation of the expression of an α-factor-degrading protease (Bar1), a regulator of G-protein signaling protein (Sst2) that stimulates Gpa1-GTP hydrolysis, and Gpa1 itself. Ste2 is also down-regulated by endocytosis, both constitutive and ligand-induced. Ste2 internalization requires its phosphorylation and subsequent ubiquitinylation by membrane-localized protein kinases (Yck1 and Yck2) and a ubiquitin ligase (Rsp5). Here we demonstrate that three different members of the α-arrestin family (Ldb19/Art1, Rod1/Art4 and Rog3/Art7) contribute to Ste2 desensitization and internalization, and do so by discrete mechanisms. We provide genetic and biochemical evidence that Ldb19 and Rod1 recruit Rsp5 to Ste2 via PPxY motifs in their C-terminal regions; by contrast, the arrestin-fold domain at the N-terminus of Rog3 is sufficient to promote adaptation. Finally, we show that Rod1 function requires calcineurin-dependent dephosphorylation.
INTRODUCTION
For survival, eukaryotic cells sense and respond to changes in external conditions using, in many cases, a G-protein-coupled receptor (GPCR) (Granier & Kobilka 2012; Katritch et al. 2013). Most clinically used pharmaceuticals act on GPCR (Shoichet & Kobilka 2012; Garland 2013). After an initial response, cells normally adapt by becoming desensitized to the stimulus, whereas chronic GPCR action can lead to inflammation and other pathophysiology (Gainetdinov et al. 2004; Kelly et al. 2009). Thus, the mechanisms underlying both GPCR signal transmission and its attenuation have important medical implications.

In S. cerevisiae, GPCR Ste2 in the plasma membrane (PM) of a MATα haploid cell binds α-factor (a 13-residue peptide) secreted by a MATα haploid cell, and a MATα haploid uses GPCR Ste3 to bind a-factor (a 12-residue prenylated peptide) released by a MATα haploid (Bardwell 2004). Engagement of these receptors by these ligands (mating pheromones) initiates a mitogen-activated protein kinase (MAPK) cascade. The activated MAPK evokes transcriptional and morphological responses that arrest cell growth in G1 phase of the cell cycle and convert the cells to gametes, which conjugate (mate) to form a MATa/MATα diploid (Bardwell 2004; Slessareva & Dohlmam 2006). Genetic and molecular analysis of this system has established many important concepts in GPCR-initiated signaling and its regulation (Dohlman & Thorner 2001; R. E. Chen & Thorner 2007; Yu et al. 2008).

Hyperactivation or prolonged signaling through the mating pheromone response pathway can cause cell death (N.-N. Zhang et al. 2006). To avoid this, α-factor also induces feedback mechanisms that down-regulate signaling in MATa cells. Paramount among these adaptation processes is up-regulation of expression of genes for a variety of negative regulators, such as MSG5, which encodes a phosphoprotein phosphatase that deactivates the terminal MAPK (Fus3) of the pathway (Doi et al. 1994; Zhan et al. 1997). However, induced negative regulators also include factors that desensitize the pathway far upstream by acting on α-factor, its receptor, or the associated heterotrimeric G-protein, preventing further GPCR-initiated signaling. For example, BAR1 encodes a protease that cleaves α-factor into two inactive fragments (Ciejejk & Thorner 1979; MacKay et al. 1988). SST2 encodes the first regulator of G-protein signaling (RGS) protein identified (Chan & Otte 1982; Dohlman et al. 1996). Binding of its N-terminal DEP domains to the cytosolic tail of Ste2 (Ballon et al. 2006) delivers Sst2 to the PM. Its C-terminal RGS domain is thus positioned to stimulate conversion of PM-localized GTP-bound Gpa1 back to its GDP state (Apanovitch et al. 1998). GDP-Gpa1 then reassociates with and blocks downstream signaling by the Ste4-Ste18 complex (Blumer & Thorner 1990; Zhou et al. 1999), which is also PM-anchored via S-palmitoylation and S-farnesylation of Ste18 (Hirschman & Jenness 1999; Manahan et al. 2000). Recoupling and squelching of Gβγ function is further promoted by mass action because GPA1 is up-regulated in response to pheromone (Roberts et al. 2000), concomitant with an enhanced rate of its N-myristoylation (Dohlman et al. 1993), a post-translational modification essential for Gpa1 PM targeting, coupling to Gβγ, and association with the receptor (Blumer & Thorner 1990; Song et al. 1996).

Termination of an agonist-initiated GPCR-mediated signal is also thought to involve ligand-induced receptor endocytosis (Wolfe & Trejo 2007; Sorkin & Zastrow 2009). It has been known for decades that both Ste2 and Ste3 undergo constitutive
internalization at a basal rate and that endocytosis is stimulated upon binding of the
cognate pheromone (Chvatchko et al. 1986; Jenness & Spatrick 1986; Zanolari &
Riezman 1991; Davis et al. 1993; Schandel & Jenness 1994). Pheromone binding
causes a conformational change that promotes phosphorylation of the Ste2 and Ste3 C-
termini (Renke et al. 1988; Q. Chen & Konopka 1996), mediated by PM-anchored casein kinase I isoforms (Yck1 and Yck2) (Feng & Davis 2000; Ballon et al. 2006).
Phosphorylation is a prelude to receptor ubiquitinylation by a PM-associated HECT
domain-containing ubiquitin ligase (E3), Rsp5 – the mammalian ortholog of Nedd4
(Dunn & Hicke 2001; Katzmann et al. 2002; Stoll et al. 2011; Kamadurai et al. 2009).
Ubiquitin attachment targets the receptors for clathrin-mediated internalization (Tan et
al. 1993; Panek et al. 1997). The resulting endosomes are delivered to the vacuole
(equivalent to a mammalian lysosome) where the receptor is degraded (Katzmann et al.
2004; Gabriely et al. 2006).

Subsequent studies have provided more detailed analysis of spatiotemporal aspects
of Ste2 (Toshima et al. 2006; Suchkov et al. 2010) and Ste3 (L. Chen & Davis 2002;
Shields et al. 2009) internalization and better delineated the phosphorylation and
ubiquitinylation sites (Roth & Davis 2000; Toshima et al. 2009). However, how Rsp5
recognizes and is recruited to these GPCRs to catalyze ubiquitinylation remained
unresolved. Discovery in yeast of a protein family, called the α-arrestins, that serve as
adaptors for Rsp5-dependent ubiquitinylation and internalization of nutrient permeases
provided a clue (Alvarez 2008; Lin et al. 2008). The S. cerevisiae genome encodes 14
currently recognized members of the α-arrestin family (Lin et al. 2008; Nikko & Pelham
2009; Hatakeyama et al. 2010; O'Donnell 2012). The name derives from homology of
their N-terminal domains to a fold found in mammalian arrestin and β-arrestins (Alvarez
2008; Patwari & Lee 2012), first identified and implicated in blocking signaling by,
respectively, rhodopsin (Kuhn & Wilden 1987) and β-adrenergic receptor (Benovic et al.
1987).

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

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

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

Pheromone-imposed growth arrest. Response to α-factor was assessed by an agar diffusion (halo) bioassay, essentially as described (Reneke et al. 1988). In brief, cells (~10^5) were plated in top agar on solid YPD or SC medium, as appropriate. On the resulting surface were laid sterile cellulose filter disks onto which an aliquot (typically, 15 µl) of an aqueous solution (1 mg/ml) of α-factor (GeneScript, Piscataway, NJ) had been aseptically spotted, and the plates incubated at 30°C for 2-4 d. To induce α-arrestin over-expression, strains containing the tripartite S. cerevisiae Gal4-human estrogen receptor-herpes simplex virus transactivator VP16 fusion protein (Gal4-ER-VP16 or GEV) (Veatch et al. 2009) and URA3-marked multi-copy (2 µm DNA) plasmids expressing from the GAL1,10 promoter the α-arrestin of interest (as a fusion to the C-terminus of GST) were grown to mid-exponential phase. The cultures were then treated with β-estradiol (20 µM final concentration) for 3 h and plated using top agar containing 200 nM β-estradiol. Samples of the same cultures were analyzed by immunoblotting (see below) to confirm α-arrestin expression.

Mating pathway activation. As one measure of pathway activation, the level of dually phosphorylated Fus3 was assessed, as follows. Strains of interest carrying a bar1Δ mutation were grown in YPD to mid-exponential phase, a sample was removed (0 time point) and immediately thereafter the culture was treated with α-factor (15 nM final concentration), and then additional samples of an equivalent number of cells were removed at 10, 30, 60 and 90 min, harvested by rapid sedimentation in a microfuge and immediately frozen in liquid N2. The cell pellets were thawed on ice, and whole-cell protein extracts were prepared by alkaline lysis followed by collection of total protein by trichloroacetic acid (TCA) precipitation (Volland et al. 1994; Westfall et al. 2008). The 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)] containing 0.1% bromophenol blue, heated at 37°C for 15 min, resolved in a slab gel by SDS-PAGE and analyzed by immunoblotting.

As an independent measure of pathway activation, induction of a FUS1prom-eGFP reporter (Patterson et al. 2010) was monitored. Strains of interest carrying a bar1Δ mutation and also containing integrated FUS1prom-eGFP were constructed (Table 1). These cells were grown to mid-exponential phase, treated with 15 nM α-factor for 2 h, and the level of GFP expression was quantified by flow cytometry using a Beckman-Coulter FC-500 Analyzer, and the resulting data were analyzed using FlowJo™ software (Tree Star, Inc.).

Immunoblotting. Equal numbers of cells from mid-exponential phase cultures were
collected by centrifugation and lysed, and total protein was collected and resolved by SDS-PAGE as described above. The proteins in the resulting slab gels were transferred electrophoretically to nitrocellulose sheets using a semi-dry transfer apparatus (Transblot SD™, Bio-Rad, Inc.). After blocking with carrier protein, the filters were incubated (generally, several h at room temperature or overnight at 4°C) with the following primary antibodies, as appropriate: rabbit polyclonal anti-GST (Sigma); rabbit polyclonal anti-phospho-ERK (Cell Signaling); goat polyclonal anti-Fus3 (Santa Cruz); mouse monoclonal anti-HA (Roche, 12CA5); mouse monoclonal anti-ubiquitin (Santa Cruz, P4D1); and, as loading control, rabbit polyclonal anti-Pgk1 (Baum et al. 1978). The resulting immune complexes were then detected by incubation, as appropriate, with infrared dye (IRDye 680/800)-labeled secondary antibodies— goat anti-mouse IgG, goat anti-rabbit IgG, or donkey anti-goat (all from Li-Cor, Lincoln, NE)—followed by visualization using an infrared imager (Odyssey™, Li-Cor). In experiments assessing the ratio of phosphorylated to total Fus3, band intensities were quantified for each time point using ImageJ software (National Institute of Health). Alternatively, in some experiments, to monitor GFP-tagged proteins, and after blocking with SuperBlock™ (Thermo Scientific, Rockford, IL), filters were incubated with rabbit polyclonal anti-GFP antibodies (Invitrogen) and the resulting immune complexes were detected with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibodies (GE Healthcare) and, to monitor GST-tagged proteins, incubated with mouse monoclonal anti-GST antibodies (Covance) and detected with horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies (GE Healthcare). The bound immune complexes were then visualized by chemiluminescence using either ECL Western Blotting Substrate™ (Pierce) or SuperSignal West Dura Extended Duration Substrate™ (Thermo Scientific, Rockford, IL) detected with Biomax XAR film (Eastman Kodak, Rochester, NY).

Protein purification. GST-Rod1 fusions were used to assess the role of the apparent PxxIxxT motif (546PQIKIE550) in this α-arrestin in mediating its association with calcineurin, as follows. Cells of yeast strain JRY11, which expresses Cna1-GFP, were transformed with pEGKG-Rod1WT or pEGKG-Rod1AQAKAA. The resulting transformants were grown to mid-exponential phase in SC-2% raffinose and then expression of the α-arrestin was induced by addition of 2% galactose (final concentration). To activate calcineurin, cells were treated with 200 mM CaCl2 (final concentration) for 10 min, then incubated for 1 h either with vehicle alone [90% ethanol and 10% aqueous Tween-20 (v/v), designated ET] or with the potent and specific calcineurin inhibitor FK506 at a final concentration of 1 µg/ml [added from a 10 mg/ml stock dissolved in ET] prior to galactose induction. After further incubation for 3.5 h, the cells were harvested and lysed by vigorous vortex mixing with glass beads in RIPA buffer [150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, 50 mM Tris-HCl (pH 7.4)] containing protease inhibitors (Bultynck et al. 2006). After brief centrifugation to remove unbroken cells and debris, the clarified extracts were incubated with glutathione-agarose beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 2 h at 4°C, washed 3 times with 500 µL RIPA buffer, and bound proteins were eluted from the beads in SDS-PAGE sample buffer, resolved by SDS-PAGE, and presence of Cna1-GFP and GST-Rod1 analyzed by immunoblotting.

To assess in vivo phosphorylation of Rod1, pEGKG-Rod1 and pEGKG-Rod1AQAKAA were introduced into BY4741 and otherwise isogenic cnb1Δ and cna1Δ cna2Δ derivatives by DNA-mediated transformation. Transformants were grown to mid-
exponential phase in SC-2% raffinose. Cells were then treated with either ET alone or 1 mg/ml FK506 in ET for 1 h, and then α-arrestin expression was induced by addition of 4% galactose (final concentration) followed by growth at 30ºC for 4 h. Ten minutes prior to harvesting, the cultures were treated with 200 mM CaCl$_2$ (final concentration) to activate calcineurin. Protein extracts were prepared by glass bead lysis in RIPA buffer containing both 600 mM NaCl (HS-RIPA) and 1 mg/ml FK506. After clarification by centrifugation, GST-Rod1 and GST-Rod1$^{\text{AQAKAA}}$ were collected from the lysates by binding to glutathione-agarose for 2 h at 4°C. The beads were washed 2 times with 500 µl HS-RIPA, and aliquots of the bead-bound proteins were incubated at 30°C for 45 min either in phosphatase buffer alone [1 mM MnCl$_2$, 10 mM NaCl, 2 mM DTT, 0.01% Brij-35, 50 mM HEPES (pH 7.5)] or in the same buffer containing 200 units of lambda phosphatase (New England BioLabs) in either the absence or presence of phosphatase inhibitors (10 mM Na$_4$P$_2$O$_7$, 10 mM NaF, 0.4 mM NaVO$_3$, 0.4 mM Na$_3$VO$_4$, and 0.1 mM glycerol-3-phosphate). Supernatant liquid was removed by aspiration, and bound protein was eluted by incubation at 37°C for 15 min in SDS-PAGE sample buffer, resolved by SDS-PAGE (6% acrylamide gel), and analyzed by immunoblotting.

Assessment of Rsp5 co-purification with α-arrestins was performed as described (O’Donnell et al. 2013). To assess the state of α-arrestin modification by ubiquitin in vivo, BJ5459 GEV cells carrying a plasmid vector (pEGKG) for the expression of GST-Ldb19 or GST-Ldb19(K486R) were grown to mid-exponential phase and induced with 20 µM β-estradiol (final concentration) for 3 h. After harvesting by centrifugation, the cells were washed and frozen in liquid N$_2$. Cell pellets were resuspended in 600 µl co-IP buffer [100 mM NaCl, 0.2% Triton X-100, 15 mM EGTA, 50 mM Tris (pH 7.4)] containing 5 mM N-ethylmaleimide (NEM) and protease inhibitors [1 tablet of cOmplete protease inhibitor cocktail (Roche Applied Science) per 15 ml] and lysed at 4°C by vigorous vortexing with ~1 g glass beads (0.5 mm; BioSpec Products). After clarification, GST-tagged proteins were recovered from equal volumes of these extracts by incubation with GST-agarose beads for 2 h at 4°C. After 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.

**In vitro ubiquitinylation.** The ability of α-arrestins to serve as substrates for Rsp5-mediated ubiquitinylation in vitro was assessed by minor modifications of previous methods (Kee et al. 2006). Briefly, GST-Rsp5 and a catalytically-inactive mutant (GST-Rsp5$^{\text{C777A}}$) were expressed in and purified from E. coli and the GST-tag removed by cleavage with commercial rhinovirus 3C protease (PreScission™, GE Healthcare). Uba1 (E1) and Ubc1 (E2) were purified from yeast as previously described (Carroll & Morgan 2005). Plasmid DNA (1 µg) of a vector (pME32) carrying the open-reading-frames for either ROD1, or ROG3 or LDB19, or cognate derivatives containing mutated versions of one or more of their P/VPxY motifs, was used as the template to generate the corresponding [$^{35}$S]Met-labeled protein by coupled in vitro transcription-translation using the TNT Quick Coupled System™ (Promega, Sunnyvale, CA). The resulting translation mixture was treated with a final concentration 10 mM NEM for 15 min at room temperature to inactivate the deubiquitinating enzymes and ubiquitin-conjugating enzymes in the rabbit reticulocyte lysate (Enquist-Newman et al. 2008). After quenching unreacted NEM with a final concentration of 20 mM di-thiothreitol (DTT), portions (42 µl)
of each in vitro translation product were added to a reaction mixture (60 μl final volume) containing the following components at the indicated final concentrations: 600 μM ubiquitin (Sigma, St. Louis, MO), 0.5 mM ATP, 220 nM Uba1 and 3 μM Ubc1. An aliquot (10 μl) was removed as the 0 time point and reaction was initiated immediately thereafter by addition of GST-Rps5 or, as a control, GST-Rsp5C777A (100 nM final concentration). Additional aliquots were removed at 5, 15, and 30 min, and each was quenched by immediate mixing with 8X concentrated SDS-PAGE sample buffer followed by incubation at 37°C for 15 min. The resulting products were resolved by SDS-PAGE (4.5% acrylamide gel). After drying the gel, the radioactive species were detected by exposure to a Phosphorimager screen for 2 h followed by visualization on a Typhoon FLA 7000™ laser scanner (GE Healthcare). In the control samples incubated with Rsp5C777A, no detectable ubiquitinylated species were observed (data not shown).

Fluorescence microscopy. Imaging of Ste2-mCherry was performed as described (Ballon et al. 2006) and imaging of Ste2-GFP was carried out as described (Prosser et al. 2011) using an inverted fluorescence microscope (Axiovert 200, Carl Zeiss GmbH, Jena, Germany) equipped with a CCD camera [Sensicam™, PCO-Tech, Inc. (formerly Cooke Corporation), Romulus, MI], an X-Cite 120 PC fluorescence illumination system (Exfo Ltd., Quebec, Canada), and a 100X (1.4 NA) Plan-Apochromat objective. The day before examining the cells, cultures of the strains to be tested were streaked at a low dilution on plates containing minimal medium (YNB), supplemented with appropriate nutrients to select for plasmid maintenance (if needed), and grown overnight at 30°C, yielding small colonies in mid-exponential growth (as judged by the presence of cells in all cell cycle stages in the population). Single colonies were suspended in 2.75 μl of the same medium on the surface of a glass slide under a cover slip immediately before imaging at room temperature using Slidebook software (v5.0.0.32, Olympus America, San Jose, CA) with identical imaging parameters (2x2 binning, 500 ms exposure) for all samples. Images were processed using ImageJ (v1.48b) with identical max and min intensity values applied to all images.
RESULTS

Ldb19, Rod1 and Rog3 negatively regulate the mating pathway. We first tested whether any of 12 of the 14 recognized yeast α-arrestins affects the function of the Ste2 GPCR. To explore this possibility, we tested derivatives of a MATa strain (BY4741) harboring an α-arrestin deletion for their response to mating pheromone using a standard agar diffusion bioassay. We did not examine Spo23 (Tez zadze et al. 2006), which is expressed only in meiotic cells (Brar et al. 2012), or Bul3, which can be expressed only by translational read-through of a stop codon situated between two adjacent open-reading-frames (Novoselova et al. 2012). We also tested strains containing deletions of both members of paralogous pairs of α-arrestins, or deletions of 9 α-arrestins in combination (9arrΔ) (Nikko & Pelham 2009). A modest but readily detectable and reproducible increase in pheromone sensitivity (as judged by the diameter of the halo of G1-arrested cells) was observed for the 9arrΔ mutant, an ldb19Δ mutant, and a rod1Δ rog3Δ double mutant (Figure 2.1A and B) [but, not rod1Δ or rog3Δ single mutants (data not shown)]. Rod1 and Rog3 share greater similarity to each other (45% identity) than to any other α-arrestin (≤30% identity), suggesting that this pair has an overlapping function. None of the other single deletions or deletions of any of the other four paralogous pairs, e.g. aly1Δ aly2Δ (Aly1/Art6 and Aly2/Art3 share 42% identity), exhibited a change in halo size (Figure 2.1A, upper panel, and B). Thus, the effects observed were specific to just three α-arrestins, Ldb19, Rod1 and Rog3.

Neither the ldb19Δ mutant nor the rod1Δ rog3Δ double mutant exhibited the same increase in halo size displayed by the 9arrΔ cells; however, ldb19Δ, rod1Δ and rog3Δ are among the deletions carried by the 9arrΔ cells. Hence, we constructed the corresponding triple mutant and, as anticipated, we found that the pheromone sensitivity of ldb19Δ rod1Δ rog3Δ cells phenocopied that of the 9arrΔ cells (Figure 2.1A, lower panel, and B). These data suggest that absence of these three α-arrestins is responsible for the observed behavior of the 9arrΔ cells. Moreover, despite the fact that these cells possess all of the previously characterized mechanisms for recovery and adaptation, these three α-arrestins clearly contribute to down-regulation of pheromone signaling. Furthermore, the fact that the effects of these mutations is additive provides evidence that Ldb19, Rod1 and Rog3 act independently of one another.

Ldb19, Rod1 and Rog3 function independently from Bar1 and Sst2. Some α-arrestins have been implicated in aspects of vesicle-mediated transport other than endocytosis (O’Donnell et al. 2010). Therefore, it was possible that lack of Ldb19, Rod1 and/or Rog3 might enhance pheromone response simply by impeding the action of known negative regulators of pheromone signaling that require membrane trafficking (such as the secreted α-factor protease Bar1/Sst1) or membrane interaction (such as the receptor- and G protein-associated RGS protein Sst2). However, compared to an otherwise isogenic bar1Δ mutant, an ldb19Δ bar1Δ double mutant, a rod1Δ rog3Δ bar1Δ triple mutant, and an ldb19Δ rod1Δ rog3Δ bar1Δ quadruple mutant exhibited significantly greater pheromone sensitivity at every concentration of α-factor tested (Figure 2.1C). Because Bar1 is absent in these cells, the observed additive behavior shows that the increase in pheromone sensitivity has nothing to do with preventing efficient Bar1 secretion or function. Likewise, compared to an otherwise isogenic sst2Δ mutant, an ldb19Δ sst2Δ double mutant, a rod1Δ rog3Δ sst2Δ triple mutant, and an ldb19Δ rod1Δ rog3Δ sst2Δ quadruple mutant exhibited greater pheromone sensitivity at every
FIGURE 2.1 Specific α-arrestins negatively regulate pheromone signaling and act independently from secreted protease Bar1 and RGS protein Sst2. (A) Pheromone-sensitivity of wild-type cells (BY4741 MATa) and otherwise isogenic derivatives containing the indicated α-arrestin deletions (9arrΔ, ldb19Δ, rod1Δ rog3Δ, aly1Δ aly2Δ, rod1Δ rog3Δ ldb19Δ) was assessed by the agar diffusion (halo) bioassay for α-factor-induced growth arrest on YPD medium (15 μg α-factor spotted on each filter disk). One representative image is shown from independent replicates (n = 4). (B) Quantification and statistical analysis of the change in halo diameter, determined as in (A), for independent replicate experiments (n = 4). The average halo diameter for control cells was set at 100% and halo sizes for each mutant were normalized to the control. Error bars, ± SEM; ** p <0.0001; * p <0.05; n.s., value not statistically significant. (C) Pheromone-sensitivity of a bar1Δ and otherwise isogenic ldb19Δ bar1Δ, rod1Δ rog3Δ bar1Δ, and rod1Δ rog3Δ ldb19Δ bar1Δ was determined, as in (A), in response to the indicated amounts of α-factor (150 ng to 15 μg). Values represent the averages of
independent replicate experiments (n = 5); errors bars, ± SEM. (D) Pheromone-sensitivity of a sst2Δ and otherwise isogenic ldb19Δ sst2Δ, rod1Δ rog3Δ sst2Δ, and rod1Δ rog3Δ ldb19Δ sst2Δ was determined, as in (A), in response to the indicated amounts of α-factor. Values represent the averages of independent replicate experiments (n = 3); errors bars, ± SEM. (E) Pheromone-sensitivity of an sst2Δ mutant carrying the GEV chimera for β-estradiol-induced expression of genes under GAL promoter control, and containing either empty vector (high-copy URA3-marked 2 µm DNA plasmid) or the same vector harboring the indicated α-arrestin (as a fusion to GST) under GAL promoter control, was determined on SC-Ura, as in (A), using 15 µg of α-factor spotted on the filter disk, after induction with β-estradiol (see Materials and Methods). (F) Confirmation of α-arrestin expression. Proteins from whole-cell extracts of the cells shown in (E) were prepared, resolved SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Data from one representative experiment (of 5 independent replicates) is shown for (E) and (F).
concentration of \( \alpha \)-factor tested (Figure 2.1D). Again, this additive effect indicates that absence of Ldb19, Rod1 and/or Rog3 does not cause increased pheromone sensitivity by interfering with Sst2 action. Because these \( \alpha \)-arrestins negatively regulate pheromone response by a mechanism(s) independent from those exerted by either Bar1 or Sst2, we used \( \textit{bar1} \Delta \) and \( \textit{sst2} \Delta \) cells as sensitized backgrounds in which to further characterize the function of Ldb19, Rod1 and Rog3.

**Rod1 and Rog3 promote adaptation.** The increased pheromone sensitivity observed when Ldb19, or Rod1 and Rog3, are absent is consistent with a role for these proteins in receptor down-regulation and/or signal dampening. If so, over-expression of such negative regulators should stimulate recovery from pheromone signaling. The agar diffusion bioassay provides a convenient means to assess desensitization because adaptation is readily monitored by examining the rate and extent of the resumption of cell growth inside the initial zone of pheromone-imposed \( G_1 \) arrest. Normally, in the absence of Sst2, once cells are exposed to pheromone, little or no adaptation is observed even after prolonged incubation (Ballon et al. 2006); only occasional papillae arise (see, for example, Figure 2.1E, \( \textit{left panel} \)), which represent rare cells with a selective advantage (they became pheromone-resistant by acquiring a spontaneous \( \textit{ste} \) mutation) (Whiteway et al. 1989). Remarkably, over-expression of either GST-Rod1 or GST-Rog3 in \( \textit{sst2} \Delta \) cells caused turbid halos diagnostic of adaptation, recovery from \( G_1 \) arrest and a return to cell growth (Figure 2.1E). Similar turbid halos have been observed when other negative regulators of pheromone response are overexpressed (Blackwell et al. 2003). Although both \( \alpha \)-arrestins are produced at an equivalent level (Figure 2.1F), the adaptation-promoting effect of GST-Rog3 was reproducibly more potent than that of GST-Rod1.

In contrast, overexpression of GST-Ldb19, or untagged Ldb19 (data not shown), in \( \textit{sst2} \Delta \) cells did not promote adaptation (Figure 2.1E). In multiple trials, GST-Ldb19 expression was always lower than that of GST-Rod1 and GST-Rog3 (Figure 2.1F). It was formally possible, therefore, that the level of GST-Ldb19 achieved was insufficient to support adaptation. However, like GST-Ldb19, other GST-tagged \( \alpha \)-arrestins that are expressed at a level comparable to or even higher than GST-Rod1 and GST-Rog3 (\( e.g. \), GST-Art5 and GST-Csr2/Art8) also failed to promote adaptation in \( \textit{sst2} \Delta \) cells (data not shown). These observations argue, first, that the adaptation-promoting effects of Rod1 and Rog3 are specific. Second, and tellingly, because \( \alpha \)-factor is present continuously in these assays, these observations indicate that Rod1 and Rog3 may act on pheromone receptor in its ligand-occupied conformation, whereas Ldb19 may be unable to do so. These data also show that Rod1 and Rog3 act at a different level and/or via a different mechanism than Ldb19.

**Absence of \( \alpha \)-arrestins and mating pathway signaling.** Pheromone-imposed \( G_1 \) arrest is one measure of mating pathway function. We used two other independent methods to confirm that absence of \( \alpha \)-arrestins leads to an enhanced pheromone response. First, under conditions where the concentration of \( \alpha \)-factor remains essentially constant (cells carried a \( \textit{bar1} \Delta \) mutation), we compared the kinetics of activation (via dual phosphorylation) of Fus3 (Elion et al. 1990), the mating pheromone response pathway-specific MAPK (R. E. Chen & Thorner 2007), after exposing otherwise wild-type and an \( \textit{ldb19} \Delta \textit{rod1} \Delta \textit{rog3} \Delta \) triple mutant to \( \alpha \)-factor. As judged by two criteria, and as observed in the halo bioassay, there was more efficacious and
sustained signaling in cells lacking the three α-arrestins than in the control cells, although the effects were relatively modest. First, FUS3 itself is known to be a pheromone-induced gene product (Roberts et al. 2000; Elion et al. 1990; Bardwell et al. 1996) and, in multiple trials, the level of Fus3 achieved in the cells lacking Ldb19, Rod1 and Rog3 was higher than in the corresponding wild-type cells (Figure 2.2A). Second, the fraction of Fus3 in its activated state was higher and more persistent in the cells lacking the three α-arrestins than in the control cells (Figure 2.2B).

Another standard used to measure pheromone response is induction of the pheromone-responsive gene, FUS1 (Trueheart et al. 1987). We quantified the expression level of an integrated FUS1 prom-eGFP reporter gene (Patterson et al. 2010) in bar1Δ cells that were otherwise wild-type or carried the ldb19Δ rod1Δ rog3Δ mutations using flow cytometry. It was shown previously that basal signaling in the mating pheromone response pathway arises largely from stochastic spontaneous dissociation of receptor-heterotrimeric G protein complexes (Hasson et al. 1994; Siekhaus & Drubin 2003). An increase in receptor level in the PM shifts the equilibrium toward complex formation and reduces basal signaling (Ballon et al. 2006; Blumer & Thorner 1990). For this reason, if the α-arrestin-deficient cells internalize Ste2 less efficiently, then basal signaling should be reduced. Consistent with this prediction, basal expression was significantly lower in cells lacking Ldb19, Rod1 and Rog3 than in control cells (Figure 2.2C). Nonetheless, after exposure to α-factor, the level of FUS1 expression achieved in the α-arrestin-deficient cells was nearly equivalent to that in the wild-type. Therefore, induction of the FUS1 reporter was ~3-fold higher in the ldb19Δ rod1Δ rog3Δ bar1Δ cells than in bar1Δ cells (Figure 2.2D). Thus, as judged by three different assays, pheromone signaling is more sustained in cells lacking Ldb19, Rod1 and Rog3 than in control cells, consistent with loss of negative regulation of the pathway.
FIGURE 2.2 Pheromone signaling is more persistent in cells lacking Ldb19, Rod1 and Rog3. (A) Cultures of a bar1Δ strain and an otherwise isogenic ldb19Δ rod1Δ rog3Δ bar1Δ strain were grown to mid-exponential phase, and then treated with 15 nM α-factor for the indicated times. Samples were withdrawn at the indicated time points, rapidly chilled on ice, and the cells collected by centrifugation, extracted and proteins in the resulting whole-cell lysates resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. A representative experiment is shown from 3 independent replicates. (B) Values represent the mean pixel intensities for the phosphorylated and total Fus3 bands, determined as in (A), from independent replicate experiments (n = 3). Error bars, ± SEM; * p >0.01. (C) Cultures of a bar1Δ FUS1prom-eGFP strain and an otherwise isogenic ldb19Δ rod1Δ rog3Δ bar1Δ FUS1prom-eGFP derivative, were grown to mid-exponential phase. Samples of these cultures were withdrawn and the distribution of fluorescent cells was determined using a FACS (Beckman-Coulter, Model FC500) in the Flow Cytometry Facility of the UC Berkeley Cancer Research Laboratory. The remainder of each culture was treated with 15 nM α-factor for 2 h and the profile of fluorescent cells in each culture was redetermined. (D) The average fold change in the level of GFP fluorescence determined from the ratio of the areas under the curves of uninduced and pheromone-induced cells of the indicated genotypes for independent replicate experiments (n = 3) performed as in (C). Error bars, ± SEM.
Efficient Ste2 internalization requires Ldb19, Rod1 and Rog3. In *S. cerevisiae*, various classes of integral polytopic PM proteins can be endocytosed by clathrin-dependent (Myers & Payne 2013) and clathrin-independent (Prosser & Wendland 2012) routes. Caveolin-like structures in yeast (“eisosomes”) (Walther et al. 2006; Moreira et al. 2012) do not appear to be sites of endocytosis (Brach et al. 2011; Buser & Drubin 2013) and may even protect cargo from internalization (Grossmann et al. 2008). The evidence that various yeast α-arrestins are necessary for efficient internalization of distinct PM-localized nutrient permeases is compelling (Lin et al. 2008; Nikko & Pelham 2009; Hatakeyama et al. 2010; Becuwe, Vieira, et al. 2012). If Ldb19, Rod1 and/or Rog3 contribute to GPCR down-regulation by any endocytic route, one would expect to detect some Ste2 accumulation at the PM in cells lacking one or more of these α-arrestins. To localize the receptor, we first examined full-length Ste2 tagged at its C-terminus with mCherry (Ballon et al. 2006). We have demonstrated that in otherwise wild-type cells and even in the absence of pheromone the bulk of the red fluorescence resides in the vacuole (Ballon et al. 2006), presumably because the maturation rate of the mCherry chromophore is slow relative to the rate of constitutive endocytosis. Indeed, in wild-type cells, the fluorescent signal was confined almost exclusively to the vacuole (Figure 2.3A, left). By contrast, in otherwise isogenic *ldb19*∆ single, *rod1*∆ *rog3*∆ double and *ldb19*∆ *rod1*∆ *rog3*∆ triple mutants, fluorescence was discernible at the PM (Figure 2.3A).

To confirm that these conclusions were not dependent on the chromophore used to monitor Ste2 localization, and to quantify the results, the same analysis was carried out using cells expressing full-length Ste2 tagged at its C-terminus with eGFP. Again, the fluorescent signal in the parental strain was confined largely to the vacuole in virtually every cell, whereas the vast majority of the 9arr∆, *ldb19*∆, *rod1*∆ *rog3*∆ and, especially, *ldb19*∆ *rod1*∆ *rog3*∆ cells exhibited readily detectable PM fluorescence (Figure 2.3B). In marked contrast, the level of Ste2-GFP fluorescence at the PM was unchanged in mutants lacking other α-arrestins, e.g. *aly1*∆ *aly2*∆ (Figure 2.3B). These visual impressions were corroborated by averaging the intensity of PM fluorescence determined by taking multiple line scans across large numbers of cells in each field (Figure 2.3C). Together, these results are consistent with Ldb19, Rod1 and Rog3 acting as negative regulators of pheromone-initiated signaling by promoting efficient Ste2 internalization.

The ubiquitin ligase Rsp5 is required for Ldb19 and Rod1 action. Aside from an N-terminal arrestin fold that mediates interaction with specific targets, a defining hallmark of an α-arrestin is that its C-terminal sequence contains multiple copies of PPxY (and/or variants thereof, such as LPxY and VPxY) (Figure 2.4A). The PPxY motifs serve as docking sites for binding three tandem WW domains (Sudol et al. 2005) present in the HECT family ubiquitin ligase (E3) Rsp5 (Gupta et al. 2007; Rotin & Kumar 2009). In this way, α-arrestins act as adaptors that link PM substrates that lack endogenous PPxY motifs to Rsp5 (Lauwers et al. 2010; Reider & Wendland 2011), which catalyzes substrate ubiquitinylation, thereby marking cargo for endocytosis (Lin et al. 2008; Nikko & Pelham 2009; Becuwe, Vieira, et al. 2012; Merhi & Andre 2012). The primary structure of Ste2 has no PPxY motif or variant thereof in its cytoplasmic loops or cytosolic tail, yet becomes ubiquitinylated in an Rsp5-dependent manner on seven Lys residues in its C-terminal tail (Ballon et al. 2006; Toshima et al. 2009; Hicke et al. 1998).
FIGURE 2.3 Increased abundance of Ste2 in the plasma membrane in cells lacking Ldb19 and/or Rod1 and Rog3. (A) Ste2-mCherry and otherwise isogenic ldb19Δ Ste2-mCherry, rod1Δ rog3Δ Ste2-mCherry, and rod1Δ rog3Δ ldb19Δ Ste2-mCherry were examined by fluorescence microscopy and representative images recorded as described in Materials and Methods. (B) As in (A), except the cells expressed an integrated copy of Ste2-GFP as the sole source of the receptor and the 9arrΔ Ste2-GFP and aly1Δ aly2Δ Ste2-GFP stains were also visualized. (C) Mean intensity of PM fluorescence was quantified for each of the indicated strains (≥50 cells each) using ImageJ and plotted in arbitrary units (a.u.). Those values significantly different than the control cells were assessed using a one-way ANOVA test with Tukey’s post hoc comparison (165). * p<0.0001.
To determine whether Rsp5 recruitment is required for negative regulation of pheromone signaling by Ldb19, Rod1 or Rog3, we first mutated the two PPxY motifs in each of these \( \alpha \)-arrestins to PAXA, and then used two different methods to assess whether these point mutations successfully abrogated interaction with Rsp5. In the case of Rog3, we also mutated its VPxY motif because an identical motif in \( \alpha \)-arrestin Rim8/Art9 was shown to mediate Rsp5 association (109). As observed for other \( \alpha \)-arrestins (Lin et al. 2008; O’Donnell et al. 2013; Merhi & Andre 2012), we found that Rsp5 efficiently co-purified with GST-Ldb19, GST-Rod1 and GST-Rog3 (and not with a GST control), whereas the corresponding PPxY-less (or, in the case of Rog3, P/VPxY-less) mutants exhibited dramatic decreases in the amount of Rsp5 recovered: Ldb19 (93% reduction); Rod1 (72% reduction); and, Rog3 (83% reduction) (Figure 2.4B). The residual amount of Rsp5 observed for the mutants does not represent residual interaction, but rather non-specific background because no further reduction below this threshold was observed when a complete C-terminal truncation [Rog3(Δ400-733)] was examined (Figure 2.4B). As an independent and more sensitive indicator of the ablation of Rsp5 interaction by these point mutations, we took advantage of the fact that this E3 efficiently ubiquitinylates endogenous Lys residues in the \( \alpha \)-arrestins (Lin et al. 2008; Nikko & Pelham 2009; Becuwe, Vieira, et al. 2012; Merhi & Andre 2012). We found that each of the three \( \alpha \)-arrestins, prepared by coupled in vitro transcription and translation, served as an efficient substrate for Rsp5-dependent ubiquitinylation, as judged by complete conversion of the starting material to a broad spectrum of poly-ubiquitinylated slower-mobility species (Figure 2.4C). In contrast, the corresponding PPxY point mutations abolished modification almost entirely (Figure 2.4C). Thus, as judged by two different criteria, the point mutants we generated in Ldb19, Rod1 and Rog3 clearly compromise their interaction with Rsp5.

We then used a complementation test to determine if Rsp5 association is required for Ldb19 function in pheromone signal dampening. As described earlier (Figure 2.1A), \( ldb19\Delta \) bar1\Delta cells display a halo of pheromone-induced growth inhibition that is larger than an isogenic bar1\Delta cells (Figure 2.5A). Re-introduction of wild-type LDB19 (expressed from its native promoter on a low-copy-number CEN plasmid) into the \( ldb19\Delta \) bar1\Delta strain reduced the halo size to that observed in the bar1\Delta strain, whereas even single PAXA point mutations and the double (“PPxY-less”) mutant were unable to do so (Figure 2.5A and B). Thus, the ability of Ldb19 to down-modulate signaling requires its interaction with Rsp5.

To determine if Rsp5 association is required for either Rod1 or Rog3 function in signal dampening, we exploited their ability to promote recovery and adaptation in sst2\Delta cells exposed to pheromone, as described earlier (Figure 2.1E). Over-expression of wild-type ROD1 (as a GST fusion from the GAL1 promoter on a high-copy-number 2 \( \mu \)m DNA plasmid) in sst2\Delta cells produced a turbid halo and reduced halo size, whereas even single PAXA point mutations and the double (“PPxY-less”) mutant were unable to do so (Figure 2.5C, top panel), even though the Rod1 mutants were expressed at a level as high or higher than the wild-type protein (Figure 2.5D). Thus, as for Ldb19, the ability of Rod1 to down-regulate signaling also requires its interaction with Rsp5.

Strikingly, as judged by the same assay, over-expression in sst2\Delta cells of wild-type Rog3, single PAXA mutants and the double PPxY-less mutant (Figure 2.5C, lower panel), and even the P/VPxY-less derivative lacking all three of its Rsp5-binding motifs
FIGURE 2.4 P/VPxY motifs in Ldb19, Rod1 and Rog3 are required for Rsp5 binding and Rsp5-mediated ubiquitylation. (A) Schematic depiction of the primary structures of Ldb19, Rod1, Rog3. Residues (numbers below each bar) comprising the arrestin fold (blue) in Ldb19 according to (Lin et al. 2008) and in Rod1 and Rog3 as predicted by the Phyre2 modeling algorithm (Kelley & Sternberg 2009) are shown, and positions of the consensus Rsp5-binding motifs, PPxY (purple) and VPxY (green), and reported ubiquitylated Lys residue(s) (black lines) are indicated. (B) Cultures of a GEV derivative of vacuolar protease-deficient strain BJ5459 expressing the indicated α-arrestin or the derived P/VPxY substitution mutant were grown to mid-exponential phase. Protein expression was induced with β-estradiol, and the cells harvested by centrifugation and ruptured by vortex with glass beads. GST fusions (pink dots) were captured by binding to glutathione-agarose beads and, the bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting. (C) The Rsp5-catalyzed and time-dependent ubiquitylation of an [35S]Met-labeled α-arrestin or its cognate PPxY substitution mutant (pink dots), prepared by coupled in vitro transcription-translation, was performed and analyzed using a phosphoimager as described in M&M.
(Figure 2.5E), produced turbid halos, even though the P/VPxY mutations eliminated the ability of Rog3 to associate with (Figure 2.4B) and be modified by Rsp5 (Figure 2.4C). Hence, in contrast to Ldb19 and Rod1, the ability of Rog3 to squelch pheromone signaling does not obligatorily require association with Rsp5. However, it was possible that Rog3 associates with its closest paralog Rod1 to form a hetero-dimer (or higher oligomer), and Rsp5 recruited by this partner overcomes the loss of the P/VPxY motifs in Rog3. However, this possibility was eliminated (as well as effects of Rog3 mediated through Ldb19) because over-expressed Rog3 and its PPxY-less derivative still promoted efficient adaptation in cells lacking endogenous Ldb19, Rod1 and Rog3 (Figure 2.5E). Thus, negative regulation of pheromone signaling by Rog3 does not require interaction, either direct or indirect, with Rsp5. Indeed, just the arrestin fold domain at the N-terminus of Rog3 is sufficient to promote desensitization because over-expression of a truncation mutant, Rog3(Δ400-733), yielded halos just as turbid, if not more so, than full-length Rog3. By contrast, Ldb19(Δ447-818) and Rod1(402-837) were non-functional (data not shown). Therefore, Rog3 can act on the receptor to negative regulate signaling by a mechanism that is independent of the other two α-arrestins and Rsp5.
FIGURE 2.5 Ldb19 and Rod1, but not Rog3, require Rsp5 binding to down-regulate pheromone signaling. (A) Pheromone sensitivity of a bar1Δ strain and an otherwise isogenic ldb19Δ bar1Δ mutant carrying either empty vector (HIS3-marked CEN plasmid) or the same vector expressing wild-type LDB19 or derivatives containing point mutations in each or both of its PPxY motifs binding was determined as in Figure 2.1A, except that the medium was SC-His. (B) Quantification and statistical analysis of the change in halo diameter, determined as in (A), for independent replicate experiments (n = 3). The average halo diameter for control cells was set at 100% and the halo sizes for each mutant were normalized to the control. Error bars, ± SEM; * p <0.0001. (C) Pheromone sensitivity of cultures of sst2Δ GEV cells overexpressing either Rod1 or Rog3, as indicated, or the derived PPxY point mutants, under control of the GAL promoter on a high-copy URA3-marked (2 µm DNA) plasmid was determined as in Figure 2.1E. (D) Proteins from whole-cell extracts of the cells shown in (C) were
prepared, resolved SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Data from one representative experiment (of 5 independent replicates) is shown for (C) and (D). (E) Pheromone sensitivity of cultures of sst2Δ GEV cells or ldb19Δ rod1Δ rog3Δ sst2Δ GEV cells overexpressing either Rog3 or the derived cognate P/VPxY point mutants, under control of the GAL promoter on a high-copy URA3-marked (2 µm DNA) plasmid was determined as in (C). (F) Confirmation of protein expression, as in (D). Data from one representative experiment (of 3 independent replicates) is shown for (E) and (F).
Ubiquitinylation of Ldb19, Rod1 and Rog3 is dispensable for signal dampening. It has been demonstrated that the cognate α-arrestin itself becomes ubiquitinylated during the process of recruiting Rsp5 to nutrient permeases (Lin et al. 2008; Nikko & Pelham 2009; O'Donnell et al. 2013; Becuwe, Vieira, et al. 2012; Merhi & Andre 2012). Moreover, the Lys residues in Ldb19 and Rod1 that are ubiquitinylated have been mapped (Figure 2.4A), and it was reported that corresponding K-to-R mutations in Ldb19 and Rod1 block endocytosis of their target nutrient permeases, the arginine transporter Can1 (Lin et al. 2008) and the lactate transporter Jen1 (Becuwe, Vieira, et al. 2012), respectively, suggesting that ubiquitinylation is required for the endocytosis-promoting function of these and other α-arrestins (Merhi & Andre 2012).

Our PPxY mutants of Ldb19, Rod1 and Rog3 should prevent ubiquitinylation of both the α-arrestin and its cargo because they cannot bind Rsp5. Hence, the failure of PPxY-less versions of Ldb19 (Figure 2.5A) and Rod1 (Figure 2.5C) to promote signal dampening could be due to defective ubiquitinylation, rather than an inability to deliver Rsp5 to the receptor (and/or other targets). To distinguish between these possibilities, we generated K-to-R mutations in the ubiquitinylated sites in Ldb19 and Rod1 to selectively disrupt their ubiquitinylation, but leave Rsp5 binding intact. First, we generated GST-Ldb19(K486R) (Lin et al. 2008) and confirmed by pull-down, SDS-PAGE analysis and immunoblotting that GST-Ldb19 is ubiquitinylated in vivo, whereas GST-Ldb19(K486R) is not (Figure 2.6A). Strikingly, as judged by the complementation test, re-introduction of either wild-type LDB19 or LDB19(K486R) into the ldb19∆ bar1∆ strain reduced the halo size to that observed in the bar1∆ strain (Figure 2.6B and C). Thus, the ability of Ldb19 to down-modulate signaling requires Rsp5 interaction (Figure 2.5A), but does not require its own ubiquitinylation.

Next, we generated GST-Rod1(K235R K245R K264R K267R), hereafter Rod1 4KR, based on published data about the ubiquitinylation sites in Rod1 (Becuwe, Vieira, et al. 2012). As assessed by the adaptation assay, over-expression of either wild-type ROD1 or ROD1 4KR in sst2∆ cells produced equally turbid halos (Figure 2.5D). Hence, the ability of Rod1 to down-regulate signaling also requires Rsp5 interaction (Figure 2.5C), but apparently not its own ubiquitinylation.

The mapped ubiquitinylated Lys residues in Rod1 are all conserved at the equivalent position in Rog3. Hence, we also generated GST-Rog3(K235R K245R K264R K267R), hereafter Rog3 4KR. As assessed by the adaptation assay, over-expression of either wild-type ROG3 or ROG3 4KR in sst2∆ cells produced turbid halos (Figure 2.5D). Thus, the ability of Rog3 to squelch signaling requires neither its Rsp5 interaction (Figure 2.5C and E) nor, apparently, its own ubiquitinylation.

Ldb19, Rod1 and Rog3 associate with the C-terminal cytosolic tail of Ste2. Extraction of the receptor from membranes requires addition of detergent (Ballon et al. 2006), which might cause misfolding and disrupt protein-protein interactions. Cumulative evidence indicates that one of the most dramatic changes that occurs in the receptor upon pheromone binding in situ is a conformational alteration that make its C-terminal extension (cytosolic "tail") more susceptible to proteolysis (Büküşoğlu & Jenness 1996), to greatly enhanced Yck1- and Yck2-dependent phosphorylation (Ballon et al. 2006; Reneke et al. 1988; Hicke et al. 1998), and to overt Rsp5-dependent ubiquitinylation at multiple sites (Hicke et al. 1998; Toshima et al. 2009).
FIGURE 2.6 Ubiquitinylation of Ldb19, Rod1 and Rog3 is not required for down-regulation of pheromone signaling. (A) Cultures of a GEV derivative of BJ5459 expressing either Ldb19 or the derived 486R substitution mutant (as a GST fusion from the GAL promoter) were grown to mid-exponential phase, induced with β-estradiol, and lysates prepared and analyzed as in Figure 2.4B, using the indicated antibodies. Cells expressing either Rod1 or the derived 4K-toR mutant were grown to mid-exponential phase in 4% raffinose, induced with 2% galactose for 3 hrs, treated with 2% dextrose for 5 min, and lysates prepared and analyzed as in Figure 2.4B. (B) Pheromone sensitivity of a bar1Δ strain and an isogenic ldb19Δ bar1Δ mutant carrying either empty vector (HIS3-marked CEN plasmid) or the same vector expressing wild-type LDB19 or the K486R substitution mutant was determined as in Figure 2.1A, except that the medium was SC-His. (C) Quantification and statistical analysis of the change in halo diameter, determined as in (B), for independent replicate experiments (n = 3). The average halo diameter for control cells was set at 100% and the halo sizes for each mutant were normalized to the control. Error bars, ± SEM; * p <0.0001. (D) Pheromone sensitivity of cultures of sst2Δ GEV cells expressing either GST-Rod1 or GST-Rog3, as indicated, or the derived 4K-toR substitution mutants, under the GAL promoter on a high-copy URA3-marked (2 µm DNA) plasmid was determined as in Figure 2.1E. One representative image (of 3 independent replicates) is shown.
These findings suggest that Ldb19, Rod1 and/or Rog3 might interact with this same region of the receptor once it becomes exposed, facilitating Rsp5-dependent modification. Hence, we examined the ability of these α-arrestins to interact with a purified soluble version of the 135-residue C-terminal extension of Ste2 [GST-Ste2(297-431)] bound to beads. Moreover, because we demonstrated (Figure 2.6) that ubiquitinylation of Ldb19, Rod1 and Rog3 is not required for their actions in vivo, it was not necessary to prepare ubiquitin-decorated versions of each molecule to analyze their ability to associate with the Ste2 tail in vitro. Indeed, we found that 35S-labeled Ldb19, Rod1 and Rog3 reproducibly bound better to the C-terminal tail of Ste2 than to the GST control (Figure 2.7A and B). Other α-arrestins (e.g., Aly1/Art6 and Art5) did not show any increase above the control (Figure 2.7A and B). Thus, Ldb19, Rod1 and Rog3 fulfill all the requirements of adaptors for the down-regulation, modification and endocytosis of Ste2; they associate specifically with Rsp5, bind preferentially to the C-terminal tail of Ste2, and reduce Ste2 abundance at the cell surface.

**Rod1 must be dephosphorylated by calcineurin to promote signal desensitization.** Endocytosis of PM nutrient transporters is regulated by metabolic conditions. For example, under glucose-replete conditions, the lactate transporter Jen1 is internalized in a Rod1- and Rsp5-dependent manner (Becuwe, Vieira, et al. 2012). Upon glucose limitation, however, Snf1 (yeast AMPK) is activated (Hedbacker & Carlson 2008) and phosphorylates Rod1 (Shinoda & Kikuchi 2007), inhibiting its function. This event allows Jen1 to accumulate in the PM (Becuwe, Vieira, et al. 2012). Similarly, we noted during the course of our studies that over-expression of Rod1 on galactose medium, another condition under which Snf1 is active (Hedbacker & Carlson 2008), failed to promote adaptation of sst2Δ cells (data not shown). These data suggest that Rod1 phosphorylation blocks its association with Ste2. Glc7 (yeast phosphoprotein phosphatase-1) bound to targeting subunit Reg1 has been implicated in the dephosphorylation of Rod1 required for Jen1 internalization (Becuwe, Vieira, et al. 2012). For several reasons, we suspected that the role of Rod1 in promoting pheromone signal desensitization might instead be a feedback mechanism that requires dephosphorylation controlled by Ca^{2+} and the Ca^{2+}-activated phosphatase calcineurin (CN; yeast phosphoprotein phosphatase-2B). First, elevated Ca^{2+} influx is a consequence of pheromone action (Ohsumi & Anraku 1985). Second, we showed previously that optimal CN function is required for efficient adaptation after MATa cells are exposed to α-factor (Cyert & Thorner 1992). Third, we demonstrated recently that CN-mediated dephosphorylation of another α-arrestin, Aly1, is required to promote endocytosis of the aspartate and glutamate transporter Dip5 (O’Donnell et al. 2013). Finally, like Aly1, Rod1 was identified in a global screen for CN substrates and found to be efficiently dephosphorylated by CN in vitro (Goldman, unpublished results).

CN is recruited to substrates that contain a conserved docking motif, i.e., PxIxIT and variants thereof (Roy & Cyert 2009). Indeed, compared to a GST control, a GFP-tagged derivative of the CN catalytic subunit Cna1 co-purified with GST-Rod1 from yeast extracts, whereas an equivalent level of GST-Rod1^{AQAKAA} (in which the sole PxIxIT motif, 545PQIKIE550, was mutated) exhibited a dramatic decrease (>90%) in the amount of Cna1-GFP recovered (Figure 2.8A). Consistent with this site being required for efficient CN-dependent dephosphorylation in vivo, we found that GST-Rod1^{AQAKAA} resolved into two bands in cells in which CN was activated by exposure to 200 mM
FIGURE 2.7 Ldb19, Rod1 and Rog3 bind preferentially to the C-terminal tail of Ste2. (A) GST or GST-Ste2(297-431) (“tail”), which was constructed, expressed in E. coli, and purified to apparent homogeneity, were used to coat glutathione-agarose beads to an equivalent level (lower panel). In this representative experiment, samples of the beads were incubated, in duplicate, with equivalent amounts (cpm) of [35S]Met-labeled molecules of the indicated α-arrestins (pink dots), prepared by coupled in vitro transcription and translation, and the amount of bound radioactive protein detected (upper panel) was quantified using a phosphoimager, as described in Materials and Methods. (B) The average fold increase in the level of radioactivity bound to the GST-Ste2tail construct, relative to that bound to the GST control, for the indicated α-arrestins in multiple independent replicate experiments (n = 3), each performed essentially as in (A). Error bars, ± SEM. Dashed line represents no change in binding when compared to the GST control. (C) Same binding experiment as in (A) except with in vitro transcribed and translated Rog3 and Rog3Δ400. (D) The average fold increase in binding as determined in (B) from multiple independent replicate experiments (n = 3).
CaCl₂, whereas GST-Rod1 migrated as a single band (Figure 2.8B, upper panel). The GST-Rod₁^{AQAKAA}-derived species were clearly phosphorylated because they collapsed to a single faster mobility band upon treatment with λ phosphatase (Figure 2.8B, upper panel). Likewise, GST-Rod1 also migrated as two distinct bands when the cells were treated with the potent CN-specific inhibitor FK506 (Figure 2.8B, upper panel), or in mutants lacking either the Ca^{2+}-binding regulatory subunit (Cnb1) of CN (Figure 2.8B, middle panel) or both of its catalytic subunit isoforms (Cna1 and Cna2) (Figure 2.8B, lower panel). These GST-Rod1-derived bands co-migrated with those observed for GST-Rod₁^{AQAKAA}. These species also collapsed to the same single faster mobility species after λ phosphatase treatment (Figure 2.8B). These findings demonstrate that efficient dephosphorylation of Rod1 in vivo requires CN action.

In agreement with the conclusion that phosphorylation of Rod1 at these CN-sensitive sites blocks its ability to promote desensitization after pheromone response, we observed less turbid halos in the adaptation assay when GST-Rod₁^{AQAKAA} was overexpressed in sst2Δ cells, compared to GST-Rod1 (Figure 2.8C). Most tellingly, in cells lacking functional CN (either cnb1Δ or cna1Δ cna2Δ mutants), overexpression of GST-Rod1 was unable to promote any detectable adaptation (Figure 2.8D). By contrast, adaptation promoted by GST-Rog3 remained unaffected (Figure 2.8D), even though GST-Rod1 and GST-Rog3 were expressed at an equivalent level in both wild-type cells and the CN-deficient mutants (Figure 2.8E). We conclude that dephosphorylation mediated by the Ca^{2+}-dependent phosphatase CN is essential for Rod1 to down-regulate pheromone signaling.
FIGURE 2.8 Rod1-mediated desensitization requires calcineurin-dependent dephosphorylation. (A) A single PxIxIT motif mediates CN-Rod1 interaction. Cultures of strain JRY11, which produces Cna1-GFP from the native CNA1 promoter at the endogenous CNA1 locus on chromosome XII, and also expressing, as indicated, either GST alone, GST-Rod1 or GST-Rod1AQAKAA, were grown to mid-exponential phase, harvested, lysed, and proteins in the resulting extracts captured on glutathione-agarose beads, resolved by SDS-PAGE and analyzed with the indicated antibodies. (B) Rod1 is phosphorylated at CN-sensitive sites. Cultures of strain BY4741 (WT) or otherwise isogenic cnb1Δ and cna1Δ cna2Δ derivatives, as indicated, expressing either GST-Rod1 or GST-Rod1AQAKAA, were grown to mid-exponential phase, stimulated with 200 mM CaCl₂ to activate CN in either the absence or presence (+) of the CN inhibitor FK506 ("FK"). After harvesting and lysis, proteins in the resulting extracts were purified.
by capture on glutathione-agarose beads (lanes 1-4). Then, samples of the material shown in lanes 1-4 were either not treated or treated (+) with lambda phosphatase ("λ"), in either the absence or presence (+) of phosphatase inhibitors ("PPase inhibitors"), and the resulting products separated under SDS-PAGE conditions that permit resolution of phospho-isoforms and analyzed with anti-GST antibodies. (C) Lack of CN binding reduces Rod1-mediated adaptation. Pheromone sensitivity sst2Δ GEV cells expressing either GST-Rod1 or GST-Rod1^{AQAKAA}, as indicated, under the GAL promoter on a high-copy URA3-marked (2 µm DNA) plasmid was determined as in Figure 2.1E. (D) Absence of CN eliminates Rod1-mediated adaptation, but not Rog3-mediated adaptation. Pheromone sensitivity of cultures of sst2Δ GEV cells and isogenic cnb1Δ and cna1Δ cna2Δ derivatives, as indicated, expressing either GST-Rod1 or GST-Rog3 under the GAL promoter on a high-copy URA3-marked (2 µm DNA) plasmid was determined as in Figure 2.1E. (E) Confirmation of protein expression. Whole-cell extracts of the cells used in panels C and D were prepared, resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. Here phospho-isoforms were not separated because different SDS-PAGE conditions were used. Data from one representative experiment (of 3 independent replicates) is shown for (C), (D) and (E).
DISCUSSION

It has been presumed that the ligand-induced phosphorylation (Reneke et al. 1988) and Rsp5-dependent ubiquitinylation of multiple Lys residues (Hicke et al. 1998; Dunn & Hicke 2001; Toshima et al. 2009) in the C-terminal cytosolic tail of Ste2, and the ensuing increased rate of receptor endocytosis (Jenness & Spatrick 1986; Davis et al. 1993), contributes to signal dampening and recovery from pheromone response. Consistent with this notion, truncations that eliminate the C-terminal tail of Ste2 prevent receptor internalization and result in increased pheromone sensitivity and marked prolongation of pheromone-imposed G₁ arrest (Reneke et al. 1988; Konopka et al. 1988). However, we now know that the C-terminal tail of Ste2 is also the primary binding site for the RGS protein Sst2 and thus required for efficient PM recruitment and function of Sst2 in deactivation of GTP-bound Gpa1 (Ballon et al. 2006). Moreover, absence of Sst2 results in an elevation in pheromone sensitivity and in a sustained pheromone response quite similar to that conferred by receptor C-terminal truncations (Reneke et al. 1988; Konopka et al. 1988), raising the formal possibility that, compared to its role in tethering Sst2 in the vicinity of its substrate, the C-terminal tail-dependent endocytosis of Ste2 per se has little or no function in post-pheromone adaptation. Contrary to that viewpoint, the effects of an sst2Δ mutation and receptor truncation are somewhat additive and high-level overexpression of SST2 can promote recovery of tail-less Ste2 mutants from the effects of pheromone (Reneke et al. 1988; Konopka et al. 1988).

The findings we describe here support the conclusion that Rsp5-dependent modification and internalization of the receptor contribute to the overall desensitization process. Most importantly, our work answers previously unresolved questions about how Ste2 is recognized by Rsp5 to promote both its basal and ligand-induced endocytosis. Given that Rsp5 associates with the PM via its N-terminal lipid-binding C2 domain (CHO & STAHELIN 2006), but interacts with the substrates it modifies via the binding of its three tandem WW domains (Sudol et al. 2005) to PPxY motifs in those targets (Lin et al. 2008; Reider & Wendland 2011), it was a conundrum as to how Rsp5 recognized Ste2 as a substrate since this receptor lacks any sequence elements resembling PPxY. The discovery that the α-arrestin family of adaptor proteins serve as intermediaries to recruit Rsp5 to nutrient permeases that also lack endogenous PPxY motifs suggested that members of the α-arrestin class of proteins might function as the molecular matchmakers for delivering Rsp5 to Ste2.

Indeed, as documented here, three of the 14 known yeast α-arrestins contribute to signal down-regulation at the receptor level as judged by multiple independent criteria. First, lack of either Ldb19 or the paralogous pair Rod1 and Rog3 increases pheromone sensitivity, even in cells that possess all of the other demonstrated mechanisms for recovery from pheromone response. Second, simultaneous absence of Ldb19, Rod1 and Rog3 results in even greater pheromone sensitivity, comparable to a cell in which 9 α-arrestins (including Ldb19, Rod1 and Rog3) are absent. Third, among all the α-arrestins tested, only Rod1, Rog3 and Ldb19 exhibited preferential binding to the site of Rsp5-dependent modification in Ste2, its cytosolic C-terminal tail. Phosphorylation of purified GST-Ste2tail with recombinant casein kinase I neither enhanced nor inhibited binding of Ldb19, Rod1 or Rog3 (A.F. O'Donnell, unpublished results), indicating that, rather than creating epitopes for recruitment of these adaptors, phosphorylation may
simply assist in locking the tail in a solvent-accessible state after it is exposed by the pheromone-induced change in receptor conformation. Fourth, in cells expressing fluorescent derivatives of Ste2, PM accumulation of the receptor occurred in \( \text{ldb}19\Delta \) cells, in \( \text{rod}1\Delta \text{rog}3\Delta \) cells, and especially in \( \text{ldb}19\Delta \text{rod}1\Delta \text{rog}3\Delta \) (and \( \text{9arr}\Delta \)) cells and was not observed in control cells or in any other single or multiple \( \alpha \)-arrestin deletion mutants.

The vast bulk of either mCherry- or eGFP-tagged Ste2 resided in the vacuole under all circumstances. This behavior suggests that a large fraction of these chimeras may be recognized as incorrectly folded (and/or improperly glycosylated) and shunted to the vacuole via the Golgi body-to-endosome quality control pathway (Reggiori et al. 2000; Pizzirusso & Chang 2004) more efficiently than they are delivered from the Golgi compartment to the PM. Therefore, the amount of these fluorescently-tagged proteins that is properly folded and inserted into the PM may be a minority of the total synthesized. Moreover, the Golgi body-to-endosome shunt is known to depend on decoration of cargo by ubiquitin (MacGurn et al. 2012), thus making it problematic to analyze the change in modification state of our Ste2 constructs due solely to the presence or absence of Ldb19 and/or Rod1 and Rog3. Nevertheless, the observed increase in the amount of tagged Ste2 at the PM in \( \text{ldb}19\Delta , \text{rod}1\Delta \text{rog}3\Delta \) and \( \text{ldb}19\Delta \text{rod}1\Delta \text{rog}3\Delta \) cells is consistent with their role in mediating the Rsp5-dependent endocytosis of the receptor.

Our results also show that the three \( \alpha \)-arrestins contribute to Ste2 down-regulation in discrete ways (Figure 2.9). First, although paralogs Rod1 and Rog3 may have some overlapping role (because a detectable phenotype was only found for the \( \text{rod}1\Delta \text{rog}3\Delta \) double mutant), the loss of Ldb19 and absence of Rod1 and Rog3 had additive effects. Second, like Ldb19, the ability of Rod1 to promote adaptation requires Rsp5 binding, whereas the function of Rog3 in adaptation does not. Third, in the absence of CN-dependent dephosphorylation, Rod1 is unable to promote adaptation, whereas Rog3 is not subject to CN-mediated regulation. The most parsimonious interpretation of our collective findings is that the three different \( \alpha \)-arrestins may act on different states of the receptor and do so under different conditions (Figure 2.9).

Ldb19 has a similar role in PM quality control in that it mediates Rsp5-dependent ubiquitinylation and endocytic removal only of misfolded Ste2. Consistent with this view, Ldb19 bound less avidly than did either Rod1 or Rog3 to the isolated Ste2 tail \textit{in vitro}, presumably because Ldb19 recognizes an additional determinant only accessible in a misfolded receptor. For example, because native Ste2 functions as a dimer (Overton et al. 2003; Chinault et al. 2004), perhaps Ldb19 associates with the tail and also a site exposed in a monomer when the dimer dissociates. In any event, a role in removal of misfolded molecules may explain why overexpression of Ldb19 was unable to enhance the rate of recovery in the adaptation assay, which is conducted under conditions where the properly-folded state of the receptor is stabilized by ligand binding. In the absence of Ldb19, unfolded receptor is not removed from the cell surface, explaining the increase in Ste2-mCherry and Ste2-GFP observed in \( \text{ldb}19\Delta \) cells. Moreover, since misfolded receptor is not removed in \( \text{ldb}19\Delta \) cells, it has a chance to refold and recouple to the G-protein, raising the concentration of receptor competent for signaling in the first place, explaining the modest increase in pheromone sensitivity displayed by \( \text{ldb}19\Delta \) cells.
FIGURE 2.9. Distinct mechanisms of Ste2 down-regulation by the α-arrestins Ldb19, Rod1 and Rog3. The α-factor receptor (Ste2), exists primarily in three conformational states. In naïve cells, Ste2 undergoes spontaneous stochastic dissociation from its cognate heterotrimeric G-protein (not shown for clarity) at a certain rate and thereby becomes destabilized. When it does so, it may misfold. Current evidence suggests that Ldb19/Art1 has a primary role in a PM quality control pathway that mediates Rsp5-dependent ubiquitinylation and endocytic removal of such misfolded PM proteins. In the presence of α-factor, Ste2 undergoes a ligand-induced conformational change that activates and dissociates its cognate G-protein. Because Rog3-imposed inhibition of pheromone signaling does not obligatorily require its association with or modification by Rsp3, it may act similarly to classical arrestin or β-arrestin, by binding to the C-terminal tail of the receptor and sterically preventing additional rounds of G-protein activation by the pheromone-bound receptor. Later during response to pheromone, Ca$^{2+}$ influx will stimulate the CN-dependent dephosphorylation of Rod1, making Rod1 now competent to mediate Rsp5-dependent ubiquitinylation and endocytic removal of the pheromone-bound receptor, a prime example of a stimulus-induced, late-stage, negative feedback control.
When MATa cells are exposed to α-factor, Ste2 undergoes a ligand-induced conformational change that activates and dissociates its cognate G-protein; but, in contrast, when the G-protein stochastically dissociates from naïve receptor in untreated cells, activated receptor is stabilized by bound pheromone. Because Rog3 potently stimulates desensitization and does not obligatorily require its association with or modification by Rsp5 to do so, it may act in a manner similar to retinal arrestin or β-arrestin (Lohse et al. 1992). Specifically, by binding to the C-terminal tail of the receptor, Rog3 may sterically prevent the pheromone-bound receptor from catalyzing additional rounds of G-protein activation. Indeed, just like mammalian arrestin and β-arrestin, which lack PPxY motifs and have no long C-terminal extension (Hatakeyama et al. 2010), the arrestin fold domain at the N-terminus of Rog3 is sufficient to promote adaptation. Although technically challenging, it will be important to determine in future experiments whether Rog3 competes with Gpa1 for receptor binding. Alternatively, Rog3 may promote the clathrin- and Sla1-dependent, but ubiquitin-independent, route of endocytosis that has been defined for membrane proteins that contain an exposed NPFx1-2D motif (Tan et al. 1996; Howard 2002; Mahadev et al. 2007). Ste2 contains a very similar sequence, 392GPFAD396, in its C-terminal tail. On the other hand, although Rog3 does not need to recruit Rsp5 to execute its role in squelching pheromone signaling, it does possess P/VPxY motifs competent to bind Rsp5. Hence, normally, Rog3 may both interfere with receptor-G-protein recoupling and mediate Rsp5-dependent ubiquitylation, thereby enhancing the efficiency of Ste2 capture by the components of the endocytic machinery that recognize ubiquitinylated cargo (Dores et al. 2009). This dual function may explain why Rog3 appears more efficacious than Rod1 in promoting adaptation when overexpressed. Moreover, Ste2 is the first target of Rog3 identified.

Our results indicate that Rod1 is a component of a negative feedback loop that ensures complete receptor clearance after a MATa cell has committed to a productive pheromone response. One hallmark of the later stages of pheromone response is a robust influx of Ca2+ (Nakajima-Shimada et al. 2000; Martin et al. 2011). This rise in intracellular Ca2+ is sufficient to activate the Ca2+-dependent phosphatase calcineurin (Withee et al. 1997). As we have demonstrated here, CN-mediated dephosphorylation makes Rod1 competent to stimulate adaptation after MATa cells are exposed to α-factor (Figure 2.9). By contrast, loss of CN activity did not prevent Rog3-promoted adaptation to pheromone, demonstrating that regulation by CN is specific to Rod1. Indeed, the PxIxIT motif in Rod1 is not conserved in Rog3. Along with Aly1 (O’Donnell et al. 2013), Rod1 is now the second yeast α-arrestin shown to be under CN regulation. Also, in C. elegans, the function of α-arrestin CNP-1/ArrD-17 requires CN-mediated dephosphorylation (Jee et al. 2012), suggesting that CN control of α-arrestin dephosphorylation is a conserved regulatory mechanism.

Snf1 negatively regulates Rod1 function in response to glucose limitation, thereby preventing endocytosis of lactate permease Jen1 (Becuwe, Vieira, et al. 2012; Shinoda & Kikuchi 2007). Similarly, AMPK phosphorylates and promotes degradation of the mammalian α-arrestin family member TXNIP, thereby increasing glucose uptake by preventing TXNIP-mediated down-regulation of the glucose transporter GLUT1 (Wu et al. 2013). Moreover, in glucose control of Rod1 action on Jen1, Reg1-bound Glc7 seems to be responsible for Rod1 dephosphorylation and likely also prevents Snf1-
mediated phosphorylation of Rod1 by deactivating Snf1 itself (Y. Zhang et al. 2011). However, we observed a requirement for CN-dependent dephosphorylation for Rod1 action on Ste2 on glucose-rich medium, a condition under which Snf1 is not activated (Hedbacker & Carlson 2008). These observations raised two important points. First, Snf1 cannot be the only protein kinase responsible for phosphorylating and inhibiting Rod1. In fact, phosphorylation seems to be a general mechanism for blocking the endocytic action of other α-arrestin family members (O'Donnell et al. 2013; Merhi & Andre 2012; MacGurn et al. 2011). Second, it is clear, at least in the case of Rod1, that the same α-arrestin is being subject to differential phospho-regulation as a means to control endocytosis of different targets in response to distinct stimuli.

Previous reports provide evidence that ubiquitinylation of Ldb19 (Lin et al. 2008) and Rod1 (Becuwe, Vieira, et al. 2012) is necessary for their function in internalizing other cargoes. We found, however, that ubiquitinylation of Ldb19, Rod1 and Rog3 themselves was dispensable for negative regulation of Ste2-initiated signaling. Moreover, compared to the wild-type proteins, we observed only modest increases in the steady state level of non-ubiquitinylatable (PPxY-less and K-to-R) variants of these three α-arrestins, suggesting that their Rsp5-mediated modification does not trigger rapid proteasome-mediated degradation. Indeed, Rsp5 is known to install K63-linked poly-ubiquitin chains on target Lys residues (Kim & Huibregtse 2009), and binding of accessory proteins, such as ESCRT-0 (Nathan et al. 2013) and ESCRT-1(Herrador et al. 2010), blocks K63-linked chain recognition by proteasomes (Nathan et al. 2013) or K63-linked chain formation on substrates (Herrador et al. 2010). Moreover, paring back of the K63-linked chains by cellular deubiquitinylating enzymes leaves mono-ubiquitinylated Lys residues (Kee et al. 2006), which are poorly recognized by the proteasome (Pickart & Cohen 2004). Although ubiquitinylation of Ldb19, Rod1 and Rog3 is not required for their function, decoration with ubiquitin might prevent Rsp5 binding. If so, ubiquitinylation of these α-arrestins themselves would provide a built-in delay timer that, after an appropriate extent of modification, eventually dissociates Rsp5 and thereby recycles this E3. Alternatively, like phosphorylation, ubiquitinylation may be yet another means to control differentially the interaction of the same α-arrestin with different targets in response to discrete signals.

Presumably, defects in the LDB19, and especially the ROD1 and ROG3 genes, were not identified in standard screens for loss-of-function mutations that confer elevated pheromone sensitivity because of their overlapping functions. Indeed, in the case of ROD1 and ROG3, we only observed a detectable phenotype in the rod1Δ rog3Δ double mutant. One reason the effects of these mutations may appear modest is that the level of these α-arrestins is quite low— Ldb19, 295 per cell; and, Rod1, 386 per cell (Rog3 was not reported) (Ghaemmaghami et al. 2003)—compared to the number of receptors on the surface of a MATa cell, ~8,000 per cell (Jenness & Spatrick 1986; Chvatchko et al. 1986). Thus, this difference in stoichiometry may explain why it has been technically difficult to observe co-localization of these molecules with Ste2 under normal cellular conditions (C. Alvaro, unpublished observations). By contrast, co-localization of the much more abundant Sst2 (5980 molecules per cell (Ghaemmaghami et al. 2003)) with Ste2 was readily observed (Ballon et al. 2006). Indeed, co-localization of Ldb19 (Lin et al. 2008) and Rod1 (Becuwe, Vieira, et al. 2012) with their respective cargoes has not been demonstrated, even when over-expressed (C. Alvaro, unpublished observations),
perhaps indicating the transient nature of α-arrestin-target interaction. Nonetheless, when the quantity of either Rod1 or Rog3 was elevated, the adaptation assay revealed that the ability of these α-arrestins to squelch signaling by the ligand-bound form of the receptor is actually quite potent.

In addition to Ste2, it is formally possible that Ldb19, Rod1 and/or Rog3 may mediate the Rsp5-dependent modification of other factors that might lead to down-regulation of a pheromone-induced signal. One study indicates that the Gα subunit (Gpa1) of the Ste2-associated heterotrimeric G protein undergoes Rsp5-mediated mono- and poly-ubiquitinylation, which reportedly down-regulates the amount of Gpa1 at the PM by diverting it to the vacuole or to the proteasome, respectively (Torres et al. 2009). Because Gpa1 holds the Gβγ (Ste4-Ste18) complex in check and prevents signal propagation, loss of any factor that contributes to Rsp5-dependent modification of Gpa1 would stabilize Gpa1, increase Gpa1 abundance at the PM, and make cells less sensitive to pheromone. Hence, Ldb19, Rod1 and Rog3 cannot be involved in the reported Rsp5-dependent modification of Gpa1 because, as we have documented here, loss of these α-arrestins makes cells more sensitive to pheromone action. Similarly, it has been noted that Ste4 (Gβ) becomes ubiquitylinated in an Rsp5-dependent manner on Lys340, but the presence or absence of this modification does not affect the rate of turnover of Ste4 and, unlike loss of Ldb19, Rod1 and Rog3, does not affect the magnitude or duration of pheromone-induced Fus3 activation (M. Zhu et al. 2011). Hence, Ldb19, Rod1 and Rog3 cannot negatively regulate pheromone response by being responsible for mediating the reported Rsp5-dependent modification of Ste4.

Taken together, our findings indicate that, in S. cerevisiae, α-arrestins Rod1, Rog3 and Ldb19 are negative regulators of the α-factor receptor (Ste2) in MATα cells. In yeast, there are other GPCRs, including the α-factor receptor (Ste3) in MATα cells, an apparent glucose sensor (Gpr1) (Lemaire et al. 2004), and an alkaline pH sensor (Rim21) (Herrador et al. 2010). It will be important to determine which α-arrestins regulate these GPCRs and whether, as observed for nutrient permeases (Nikko & Pelham 2009; Zhao et al. 2013), there is both specificity and redundancy in which α-arrestins modulate these targets, as we have found for Ste2.

Since the time we first reported our initial observations about the apparent functions of Ldb19, Rod1 and Rog3 in contributing to down-regulation of GPCR-initiated signaling in yeast, members of the α-arrestin family in animal cells have been implicated in interacting with, modifying, and/or promoting the desensitization and endocytosis of several classes of GPCRs (Patwari & Lee 2012; Nabhan et al. 2010; Shea et al. 2012; Han et al. 2013; Kwon et al. 2013). However, it has been reported previously that β-arrestins fulfill this role (Claing et al. 2002; Callander et al. 2009) and, furthermore, the molecular mechanisms by which mammalian α-arrestins may contribute to down-regulation of GPCR signaling is currently in dispute (Nabhan et al. 2010; Han et al. 2013). Because S. cerevisiae lacks any β-arrestin homolog, our results indicate that α-arrestins alone are capable of promoting GPCR internalization. Thus, our studies in a model organism have helped to resolve an important biological question. Therefore, α-arrestin-mediated down-regulation of GPCR-initiated signaling is likely a conserved regulatory mechanism in eukaryotes. In S. cerevisiae, there are currently 14 documented α-arrestin members (Becuwe, Herrador, et al. 2012; Merhi & Andre 2012;
O'Donnell 2012), whereas, to date, only eight (ArrDC1-ArrDC5, TXNIP and possibly DSCR3 and RGP1) are recognized in animal cells (Aubry et al. 2009). Moreover, the number of mammalian GPCRs is very large. Hence, it is highly likely that many more mammalian α-arrestins remain to be identified and characterized, and their targets delineated.

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We thank Hugh Pelham (LMB, MRC, Cambridge, UK) for the 9arrΔ strain, John Hulbregtse (Univ. of Texas, Austin) for GST-Rsp5 expression constructs, Mary Matyskiela and Andreas Martin (UC Berkeley) for the gift of purified Uba1 (E1) and Ubc1 (E2) and assistance with GST-Rsp5 purification, and Greg Barton (UC Berkeley) for FlowJo™ software and assistance with FACS analysis, and Scott Emr (Cornell Univ.) and Sébastien Léon (Institut Jacques Monod, Paris) for helpful discussions.
Table 1. Yeast strains used in this study.

<table>
<thead>
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<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td><strong>MAT</strong> leu2Δ0 ura3Δ0 his3Δ1 met15Δ0</td>
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</tr>
<tr>
<td>9arrΔ</td>
<td>ecm21Δ::KanMX csr2Δ::KanMX bsd2Δ rog3Δ::NatMX rod1Δ ygr068cΔ aly2Δ aly1Δ ldb19Δ ylr392cΔ::HIS3 his3 leu2Δ0 ura3Δ0</td>
<td>(Nikko &amp; Pelham 2009)</td>
</tr>
<tr>
<td>ldb19Δ</td>
<td><strong>MAT</strong> leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 ldb19Δ::KanMX</td>
<td>Yeast deletion collection (Open Biosystems, Inc.)</td>
</tr>
<tr>
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<td>This study</td>
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<td>9arrΔ STE2-GFP</td>
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<td>Strain</td>
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<td>rod1Δ rog3Δ ldb19Δ STE2-GFP (JT6761)</td>
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<td>bar1Δ FUS1prom-eGFP (JT6686)</td>
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<td>rod1Δ rog3Δ ldb19Δ bar1Δ FUS1prom-eGFP (JT6688)</td>
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<tr>
<td>BJ5459</td>
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<td>(Jones 2011)</td>
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<td>cnb1Δ GEV (JT6694)</td>
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<tr>
<td>cna1Δ cna2Δ GEV (JT6695)</td>
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</table>

aTo generate strains with the integrated FUS1prom-eGFP reporter, this cassette (Patterson et al. 2010) was amplified by PCR and introduced by DNA-mediated transformation into a bar1Δ derivative of BY4741. The resulting strain (JT6686) was mated to a MATα ldb19Δ rod1Δ rog3Δ bar1Δ mutant. The resulting diploids were sporulated, and MATα ldb19Δ rod1Δ rog3Δ ldb19Δ bar1Δ FUS1prom-eGFP spores were
identified after tetrad dissection.

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, introduced into the cells of interest by DNA-mediated transformation and nourseothricin (NAT)-resistant colonies were selected, in which GEV (expressed under control of an ACT1 promoter) is integrated at the leu2Δ0 locus.
### TABLE 2. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Description/Reference</th>
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<tbody>
<tr>
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<td>\textit{GAL1}_{prom}^\sim \textit{GST} 2\mu, URA3</td>
<td>(Mitchell et al. 1993)</td>
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<td>pEGKG-Rod1</td>
<td>\textit{GAL1}_{prom}^\sim \textit{GST} 2\mu, URA3</td>
<td>(H. Zhu et al. 2000)</td>
</tr>
<tr>
<td>pEGKG-Rog3</td>
<td>\textit{GAL1}_{prom}^\sim \textit{GST} 2\mu, URA3</td>
<td>(H. Zhu et al. 2000)</td>
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<td>pEGKG-Ldb19</td>
<td>\textit{GAL1}_{prom}^\sim \textit{GST} 2\mu, URA3</td>
<td>(H. Zhu et al. 2000)</td>
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<td>pEGKG-Art5</td>
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<td>(H. Zhu et al. 2000)</td>
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<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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<td>Description</td>
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<td>Origins</td>
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<td><strong>a</strong>pEGKG-Rog33400 (pJT4983)</td>
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<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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<td>pRS313</td>
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<td>(Sikorski &amp; Hieter 1989)</td>
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<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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<td>pGEX4T1-GST-Ste2&lt;sup&gt;497-431&lt;/sup&gt;</td>
<td>T7, AMP</td>
<td>(Ballon et al. 2006)</td>
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</table>

<sup>a</sup>Generated by site-directed mutagenesis with synthetic oligonucleotides containing the desired codon alterations (using wildtype sequence in pRS313 vectors as the template). DNA from the corresponding gene was amplified from genomic DNA by PCR and then cloned into pEGKG.

<sup>b</sup>DNA of the corresponding gene was amplified from genomic DNA by PCR and then cloned into the *XmaI-NotI* sites in pRS313 (Sikorski & Hieter 1989).

<sup>c</sup>Generated by site-directed mutagenesis with synthetic oligonucleotides containing the desired codon alterations and the corresponding *LDB19* DNA inserted into pRS313 as the template.
CHAPTER 3: Differential Phosphorylation Regulates the Function of the \( \alpha \)-Arrestin Rod1

ABSTRACT

G-protein-coupled receptors (GPCRs) are integral membrane proteins that initiate stimulus-dependent activation of cognate heterotrimeric G-proteins and 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 \( S. \) \textit{cerevisiae} that mediates response of \( MATa \) cells to the peptide mating pheromone \( \alpha \)-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 \( \alpha \)-arrestin Rod1/Art4, which recruits the HECT-domain ubiquitin ligase Rsp5, allowing for ubiquitinylation 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 [Alvaro CG et al. (2014) \textit{Mol. Cell. Biol.}] . Here, we show that negative regulation of Rod1 by phosphorylation is mediated by two distinct stress-activated protein kinases, Snf1/AMPK and Ypk1/SGK1, and demonstrate both \textit{in vitro} and \textit{in vivo} how this phospho-regulation affects the ability of Rod1 to promote mating pathway desensitization. These studies also revealed that, in the absence of its phosphorylation, Rod1 promotes adaptation independently of Rsp5-mediated receptor ubiquitinylation, consistent with recent evidence that \( \alpha \)-arrestins can contribute to cargo recognition by both clathrin-dependent and clathrin-independent mechanisms. However, in cells lacking a component (the formin Bni1) required for the clathrin-independent route, derivatives of Rod1 that are largely unphosphorylated and unable to associate with Rsp5 still promote efficient adaptation, indicating a third mechanism by which this \( \alpha \)-arrestin promotes desensitization of the pheromone-response pathway.
INTRODUCTION

A cell must adapt rapidly to external stimuli or changes in its environment. One mechanism by which such responses are achieved is through remodeling of the repertoire of integral membrane proteins displayed in its 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 ubiquitinylation of the target protein (Horák, 2003; Dupré et al., 2004; Nikko & 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 & 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 & Trejo, 2013; Irann ejad et al., 2015). Aberrant GPCR signaling and dysregulation have been implicated in many pathophysiologies, including cancers, asthma, hypertension, neurological disorders and autoimmune diseases (O'Hayre et al, 2014; West & Hanyaloglu, 2015). For these reasons, GPCRs are the targets of the majority of clinically used pharmaceuticals (Shoichet & Kobilka, 2012; Zhang & 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 (S. cerevisiae) that mediate its response to peptide mating pheromones (Hao et al., 2013; Merlini et al., 2013).

It has been amply demonstrated that both basal and agonist-induced internalization of Ste2 (the GPCR on MATα cells that binds the mating pheromone α-factor) and Ste3 (the GPCR on MATα cells that binds the mating pheromone a-factor) require ubiquitinylation on Lys residues in their cytosolic tails and that Rsp5 (yeast ortholog of mammalian Nedd4L) is the ubiquitin ligase (E3) responsible for this modification (Dunn & Hicke, 2001; Rotin & Kumar, 2009; Ballon et al., 2006). Rsp5 catalyzes formation of K63-linked polyubiquitin chains on its substrates (Galan & Haguenauer-Tsapis, 1997; Kim & Huibregtse, 2009; Lauwers et al., 2009) leading to their recruitment into clathrin-coated pits and internalization (Weinberg & Drubin, 2012; Myers et al., 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 & Haguenauer-Tsapis, 2009; Nikko & Pelham, 2009), a family of proteins found in all eukaryotes from yeast to humans (Alvarez, 2008; Aubry & 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 & 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, 2015b) and PPxY motifs located in their C-terminal region associate with a WW domain-containing HECT-type E3 (Rotin & 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 & Pelham, 2009; O'Donnell, et al., 2010; Becuwe et al., 2012; Merhi & Andre, 2012; O'Donnell et al., 2015), and 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—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 & Pelham, 2009; Lauwers et., 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; Zhao et al., 2013; O'Donnell 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 & Kikuchi, 2007; MacGurn et al., 2011; Becuwe et al., 2012; Jee et al., 2012; Merhi & Andre, 2012; O'Donnell et al., 2013; Alvaro et al., 2014; Herrador et al., 2015). The latter 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.

In MATa cells, three α-arrestins contribute to desensitization of the mating pheromone response pathway by mediating internalization of Ste2 (Alvaro et al., 2014). Our findings indicated, first, that 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, we found that Rod1/Art4 and its closest paralog Rog3/Art7, normally 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 nonetheless able to promote adaptation. Conversely, the ability of Rod1 to promote adaptation required its dephosphorylation by the Ca²⁺/calmodulin-stimulated phosphoprotein phosphatase calcineurin, whereas Rog3 did not. These observations focused our attention on investigating the underlying mechanisms involved in the phospho-regulation of Rod1. As described here, we have 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 & METHODS

Strains and growth conditions. Yeast strains (Table 1) were grown at 30°C in either rich (YPD) or synthetic complete (SC) medium containing 2% glucose (unless another carbon source is specified) and with appropriate nutrients to maintain selection for plasmids, if present (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 2012). Briefly, DNA amplification by the polymerase chain reaction employed Phusion™ DNA polymerase (New England Biolabs, Ipswich, MA) and all constructs were verified by 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 µl) of an aqueous solution (1 mg/ml) of synthetic α-factor (GeneScript, Piscataway, NJ) was aseptically spotted, and the plates were incubated at 30°C for 4 to 5 days. In those experiments in which α-arrestin overexpression 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 µm DNA) plasmid expressing from a GAL promoter the α-arrestin of interest [which was fused to the C-terminus of glutathione S-transferase (GST)] were grown to mid-exponential phase treated with β-estradiol (20 µM final concentration) for 3 h, 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 mid-exponential phase cultures were collected by centrifugation and stored at -80°C. The cell pellets were thawed on ice, and whole-cell protein extracts were prepared by alkaline lysis followed by collection of total protein by trichloroacetic acid (TCA) precipitation (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°C 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 µl of calf intestinal phosphatase (CIP) (10,000 units/ml) for 1 h at 37°C. 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 semi-dry transfer apparatus (Transblot SD; Bio-Rad, Inc.). After blocking with carrier protein, the filters were incubated (generally overnight at 4°C) with one of the following primary antibodies: rabbit polyclonal anti-GST (Sigma), rabbit polyclonal anti-Rsp5 (gift of Dr. Allyson F. O'Donnell), or rabbit polyclonal anti-Pgk1 (this laboratory), as a loading control. The resulting immune complexes were then detected by incubation, as appropriate, 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 *E. 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 \(A_{600nm} = 0.6\), and protein expression was induced by the addition of isopropyl-\(\beta\)-D-thiogalactopyranoside (final concentration 0.5 mM). After aeration for 5 h at 37°C, cells were harvested and the GST fusion protein was purified by column chromatography on glutathione-agarose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The beads were washed 3 times with 500 µl lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol [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 Univ.) or purified analog-sensitive Ypk1(L424A) (gift of Dr. Alexander Muir, this laboratory) was incubated at 30°C in protein kinase assay buffer (20 mM Tris-HCl, pH 7.2, 125 mM potassium acetate, 12 mM MgCl\(_2\), 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT, 0.02% BSA, 25 mM β-glycerol phosphate, and 1 mM sodium orthovanadate) with 100 µM γ-[\(^{32}\)P]ATP (5 × 10\(^5\) cpm/nmol) and 0.5 µg 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 µM 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 \(\alpha\)-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 mid-exponential phase and induced with β-estradiol (20 mM final concentration) for 3 h. After harvesting by centrifugation, cells were washed and frozen in liquid N\(_2\). Cell pellets were resuspended in 600 ml 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 (NEM) and protease inhibitors [1 tablet of cOmplete protease inhibitor cocktail (Roche Applied Science) per 15 ml] and lysed at 4°C by vigorous vortexing with ~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 h at 4°C. 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.
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 non-fermentative conditions (Fraenkel, 2003); however, when the supply of glucose is exhausted and oxygen is present, the cells can utilize non-fermentable 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 & Kikuchi, 2007; Becuwe et al., 2012), a protein kinase strongly activated under glucose-limiting conditions (Rubenstein & Schmidt, 2007; Hedbacker & 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 non-metabolizable 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 & 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 over-expression to promote adaptation on medium containing glucose versus 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 & 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 MAPKKKK 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 sst2Δ* cells grown on glucose, the halo displayed a faint, but readily detectable, turbidity compared to control cells expressing GST alone, as expected (Figure 3.1A, 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 at all was observed when
GST-Rod1 was overexpressed (Figure 3.1B, 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, $\Phi_3R/K_4S_5\Phi$ (where $\Phi$ is a hydrophobic residue) (Hardie DG 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 3.1B). The most N-terminal site is located within the arrestin fold (predicted using Phyre2.0), whereas the remaining five are found within or flanking the PPxY motifs in the C-terminal half of Rod1 (Figure 3.1B). 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, 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 3.2A). Furthermore, one of these same sites (S447) was shown to be phosphorylated by Snf1 in vitro (Shinoda & 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 & 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 non-phosphorylatable (Ala) residue or to a phospho-mimetic (Glu) residue. We found that, when overexpressed in our MAT\textit{a} sst2\Delta tester cells, Rod1(S315A S447A S641A S706A S720A S781A), henceforth abbreviated Rod1\textsuperscript{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 3.1C). In marked contrast, the Rod1(S315E S447E S641E S706E S720E S781E), henceforth abbreviated Rod1\textsuperscript{6E}, was unable to stimulate any detectable adaptation on either carbon source (Figure 3.1C). 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.
Figure 3.1 Snf1 phosphorylates Rod1 in vivo and in vitro. (A) Pheromone sensitivity of *MATa sst2Δ* stain (JT6674) carrying the GEV chimera for β-estradiol-induced expression of genes under GAL promoter control containing Rod1 as a N-terminal GST-fusion. Plated on either 2% dextrose or 2% galactose SC-Ura, using 15μg of a-factor spotted on a filter disk after induction with b-estradiol (see Materials and Methods). Pictures were taken after 4 days of incubation at 30°C. (B) Schematic of Rod1. Arrestin fold shown in blue and Rsp5-binding motifs in purple. The six putative phosphorylation sites (based on the Snf1 phosphorylation amino acid consensus motif, ΦxR/KxxSxxxΦ) are shown in green. (C) Same as in A, except with the addition of the non-phosphorylatable mutant (Rod16A) and phospho-mimetic (Rod16SE). (D) Cells from C were harvested prior to plating and whole cell extracts were prepared. Extracts were split and one fraction was then treated with calf intestinal phosphatase (CIP). The extracts were resolved by SDS-PAGE and immunoblotted. (E) Wildtype (wt) and non-phosphorylatable (6A) GST-Rod1 arrestin fold (1-402) and GST-Rod1 C-terminal extension (402-837) were purified from *E. coli* and incubated with [γ-32P]ATP and Snf1, purified from *S. cerevisiae*. The products were then resolved truncated constructs in *E. coli*, either the arrestin fold (residues 1-402) or C-terminal tail (residues 402-837). A kinase assay with purified Snf1 was performed, run on SDS-PAGE gel, and detected with an autoradiogram. Pink dots correspond to the major full length species.
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 3.1D).

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 \textit{in vitro} (Figure 3.1E). Interestingly, \textit{in vitro}, we found that Snf1-mediated phosphorylation of Rod1 occurs primarily on its C-terminal sites, and not on the one site (S315) in its arrestin fold domain, suggesting that, when it occurs \textit{in vivo}, Snf1-mediated phosphorylation may block Rod1 function mainly by impeding its recruitment of Rsp5 rather than by preventing its association with Ste2.

Three single-site mutants, Rod1(S447A), Rod1(706A) and Rod1(720A), displayed a slightly enhanced ability to promote adaptation, as compared to wild-type Rod1, whereas Rod1(315A), Rod1(S641A) and Rod1(781A) did not (Fig. 3.2B). Combining together as few as two of the mutations that had a detectable effect led to an at least additive improvement in its adaptation-promoting ability; for example, Rod1(S447A S706A) was somewhat more effective in promoting adaptation than Rod1(S447A S641A) (Fig. 3.2B). 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 progressively and dramatically increased (Figure 3.2B). Again, these differences could not be attributed to differences in the level of expression of these proteins (Figure 3.2C). Taken together, these data demonstrated that phosphorylation of all six Snf1 sites occurs \textit{in vivo} (albeit perhaps with different efficiencies at the different sites) and that, collectively, these modifications interfere with the ability of Rod1 to down-regulate Ste2.

One interpretation of our findings is that Snf1 is active at a physiologically relevant level even on glucose medium (although we cannot rule out that, during the protracted time required for growth of the lawn, the glucose concentration may become depleted to a sufficiently low level to permit Snf1 activation). 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-isoforms (or other modifications) (Fig. 3.1D). Treatment with phosphatase (CIP) collapsed these species to a single sharp band that co-migrated with Rod1^{6A} (and the mobility of Rod1^{6A} was not significantly affected by CIP treatment) (Figure 3.1D). 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 3.1D). 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 \textit{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 & 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).
Figure 3.2 Putative Snf1 phosphorylation sites in Rod1. (A) ClustalW alignment of fungal homologs of Rod1p. *S. bay* A – *S. bayanus* (Cliften et al., 2003), *S. mik* A – *S. mikatae*, (Cliften et al., 2003), *S. par* – *S. paradoxus* (Cliften et al., 2003), *S. bay* B – *S. bayanus* (Kellis, Patterson, Endrizzi, Birren, & Lander, 2003), *S. cas* – *S. castellii* (Kellis et al., 2003), *S. mik* – *S. mikatae* (Kellis et al., 2003). Black boxes highlight presides Snf1 sites. Highlight code: yellow – identical residues, pink – strong similarity in residues, green – weak similarity in residues. (B) Adaptation-post pheromone as described in Figure 3.1A. (3A – S447A, S641A, S706A; 4A – S315A, S447A, S641A, S706A; 5A – S315A, S447A, S641A, S706A, 720A; 6A – S315A, S447A, S641A, S706A, S720A, S781A). (C) Confirmation of a-arrestin expression. Cells from B were harvested prior to plating and whole cell extracts were prepared. The extracts were resolved by SDS-PAGE and immunoblotted with α-GST or α-Pgk1 (loading control).
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 protein kinase primarily responsible for negative regulatory phosphorylation of Rod1. First, if Snf1 was the major protein kinase controlling Rod1 activity on glucose, then, in a snf1Δ mutant, wild-type Rod1 would remain unphosphorylated and, when overexpressed, should be just as potent at promoting adaptation on glucose medium as Rod16A. However, that was clearly not the case (Figure 3.3). Snf1 is the founding member of a sub-family of protein kinases, present in both yeast and mammalian cells (Alessi et al., 2006; Rubenstein & Schmidt, 2007), that includes closely related enzymes called AMPK-like protein kinases (AMPKLs). In S. cerevisiae, the AMPKLs are the paralogous sets Kin1 and Kin2, Frk1 and Kin4, and Hsl1, Gin4 and Kcc4. We reasoned that if any one AMPKL was responsible for phosphorylation of Rod1 at its Snf1 sites when cell 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 Rod16A. However, in every case, Rod16A was significantly more efficacious at promoting adaptation than wild-type Rod1 in kin1Δ, kin2Δ, frk1Δ, kin4Δ, hsl1Δ, gin4Δ and kcc4Δ cells (Figure 3.3). Of course, one or more of the AMPKLs may act redundantly 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 AMPKLs (Asano et al., 2006; Szkotnicki et al., 2008). Hence, as an alternative to constructing strains carrying a snf1Δ mutation and all possible combinations of AMPKL loss-of-function mutations, we examined an elm1Δ tos3Δ sak1Δ triple mutant. Again, we found that Rod16A was more efficacious at promoting adaptation than wild-type Rod1 in the elm1Δ tos3Δ sak1Δ sst2Δ (Figure 3.3). 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 Rod16A already lacks phosphorylation at all of its Snf1 sites, its potency in promoting adaptation is lost almost completely in calcineurin (CN)-deficient cells (see Figure 3.6), 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 TORC2-activated protein kinase Ypk1 (Muir et al., 2014).
Figure 3.3 AMPK screen for novel Rod1 regulators. (A) Adaptation-post pheromone as described in Figure 3.1A in strain backgrounds lacking annotated AMPK.
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., 2015). Hence, it was an intriguing possibility that, through effects on the function of α-arrestins, that TORC2-Ypk1 signaling may also contribute to regulating the protein composition of the PM. Like Snf1, Ypk1 has a well-defined phospho-acceptor site motif, $\text{RxRxxS(Φ)}$ (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 3.4A). Genome-wide proteomic analyses (Gnad et al., 2009; Swaney et al., 2013) indicate that both sites are phosphorylated \textit{in vivo} and both sites are conserved in other \textit{sensu stricto} Saccharomyces species (Figure 3.5).

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 3.4B). Conversely, Rod1 2E was unable to promote any detectable desensitization, quite comparable to the large clear halo observed for the control (GST alone) cells (Figure 3.4B). 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 3.4C). 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 (Fig. 3.1D and 3.4D), 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 confirm that phosphorylation is occurring independently at both the Ypk1 and Snf1 sites \textit{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 \textit{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 confirmed this result, and also found that, although a fragment of Rod1 containing its N-terminal Ypk1 site is phosphorylated much less efficiently, it is also phosphorylated in a Ypk1-dependent manner (Figure 3.4E).

If Snf1- (and/or AMPKL-) and Ypk1-dependent phosphorylation both contribute to negative regulation of the desensitization-promoting function of Rod1, combination of the Rod1 6A and Rod1 2A alleles should generate a molecule whose potency 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 3.4B and 3.6A). These data corroborate genetically that
**Figure 3.4 Ypk1 phosphorylates Rod1 *in vivo* and *in vitro*.** (A) Schematic of Rod1. Arrestin fold shown in blue and Rsp5-binding motifs in purple. The six putative Snf1 phosphorylation sites are shown in green and the two putative Ypk1 phosphorylation sites (based on the Snf1 phosphorylation amino acid consensus motif, RxRxSx) are shown in pink. (B) Adaptation-post pheromone as described in Figure 3.1A with the non-phosphorylatable Ypk1 site mutant (Rod12A) and phospho-mimetic Ypk1 site mutant (Rod12E). Rod18A has all the predicted Ypk1 and Snf1 sites mutated to alanine. (C) Confirmation of α-arrestin expression and phospho-mobility analysis as described in Figure 3.2C. (D) Cells from B were harvested prior to plating and whole cell extracts
were prepared and treated with CIP as described in Figure 3.1D. These samples were run on a SDS-PAGE gel containing a lower concentration of poly-acrylamide to exaggerate band shifts. (E) GST-Rod1 arrestin fold (1-402) and GST-Rod1 C-terminal extension (402-837) were purified from *E. coli* and incubated with \(^{32}\text{P} \text{ATP}\) and an analog sensitive (as) version of Ypk1, purified from *S. cerevisiae*. Ypk1\(^{\text{as}}\) was inhibited by adding the drug, 3-MB-PP1, in designated samples. The products were then resolved and analyzed as described in Figure 3.1E. Pink dots correspond to the major full length of the GST-Rod1 fragment.
Figure 3.5 Putative Ypk1 phosphorylation sites in Rod1. (A) ClustalW alignment of fungal homologs of Rod1p. *S. bay* A – *S. bayanus* (Cliften et al., 2003), *S. mik* A – *S. mikatae*, (Cliften et al., 2003), *S. par* – *S. paradoxus* (Cliften et al., 2003), *S. bay B* – *S. bayanus* (Kellis et al., 2003), *S. cas* – *S. castellii* (Kellis et al., 2003), *S. mik* – *S. mikatae* (Kellis et al., 2003). Black boxes highlight presides Snf1 sites. Highlight code: yellow – identical residues, pink – strong similarity in residues, green – weak similarity in residues.
phosphorylation by both Ypk1 and Snf1- (and/or AMPKL-) phosphorylate and inhibit 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 yet other sites in Rod1 are phosphorylated in vivo. Consistent with this, even the Rod18A derivative displays a small, but detectable, trail of slower mobility isoforms that are removed upon CIP treatment (Figure 3.4B); nonetheless, in the Rod18A 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 (cna1Δ cna2Δ) or their shared Ca2+-binding regulatory subunit (cnb1Δ) fails to display any detectable recovery (Figure 3.6A) and, based on electrophoretic mobility smearing, Rod1 clearly remains more heavily phosphorylated in cells lacking CN than in wild-type cells (Figure 3.6B), as we showed before (Alvaro et al., 2014). Remarkably, the Rod12A mutant was able to promote faint, but detectable, halo fill-in in cells lacking CN, whereas Rod16A was barely effective at promoting adaptation in CN-deficient cells (Figure 3.6A), even though Rod12A remained more heavily phosphorylated than Rod16A in cells lacking CN (Figure 3.6B). More striking still, the Rod18A mutant was substantially more potent at promoting adaptation in CN-deficient cells than either Rod12A or Rod16A (Figure 3.6A). 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 more important to alleviate Rod1 inhibition than dephosphorylation at the latter.

As assessed by electrophoretic mobility, the sites removed from Rod18A bypass the need for CN-mediated dephosphorylation (Figure 3.6B). However, as efficacious as Rod18A is in promoting recovery in CN-deficient cells, Rod18A overexpression is even more potent in promoting adaptation in wild-type cells, where other cellular phosphatases can act in conjunction with CN (Figure 3.6A). This finding indicates that, even though the Ypk1 and Snf1 sites are clearly major points of control, Rod18A is subject to additional negative regulatory phosphorylation, consistent with our observation that, in wild-type cells, Rod18A displays a small, but detectable, trail of slower mobility isoforms that are removed upon CIP treatment (Figure 3.4D).

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 as primary and secondary docking sites for the binding of CN to its target protein (Grigoriu et al., 2013). In this regard, Rod1 possesses matches to both sequences: 545-PQIKIE-550 and 688-LLPLP-692. We demonstrated before that a corresponding Rod1AQAKAA mutant in the apparent PxIxIT site is no longer able to bind CN (Alvaro et al., 2014).
Figure 3.6 Hyperactive Rod1 bypasses Calcineurin-dependent dephosphorylation requirement. (A) Adaptation-post pheromone as described in Figure 3.1A in strain backgrounds lacking either the catalytic subunits (cna1Δ cna2Δ) or the regulatory subunit (cnb1Δ) of calcineurin. (B) Confirmation of α-arrestin expression and phosphomobility analysis as described in Figure 3.2C.
**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 three PPxY sites in its C-terminal half: PPNY, VPSY and PPAY (Figure 3.1B). We previously showed that, in otherwise wild-type MATα cells growing in glucose medium, that mutants lacking either the first, the third, or both sites (Rod1PANA, Rod1PAAA, and Rod1PPxY-less) were, unlike wild-type Rod1, incapable of promoting adaptation (Alvaro et al., 2014). Moreover, compared to wild-type Rod1, GST-Rod1PPxY-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 ubiquitinylation assays (Alvaro 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, Rod12A, Rod16A and Rod18A 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 Rod12A, Rod16A or Rod18A requires intact PPxY motifs. Quite unexpectedly, we found that derivatives of Rod12A, Rod16A and Rod18A in which all three PPxY motifs were mutated (PANA, VASA and PAAA), hereafter Rod1V/PPxY-less, retained their ability to promote adaptation more robustly than wild-type Rod1 (Figure 3.7A). These properties were not due to any differences in the level of expression of these proteins (Figure 3.7B). The reason we used the Rod1V/PPxY-less triple mutant is because when we combined the most extreme hyperactive Rod1 mutant (Rod18A) with the PANA PAAA double mutation still retained its hyperactivity compared to either Rod18A and wild-type Rod1 (data not shown), suggesting that the VPSY site might be sufficient to recruit Rsp5. However, if anything, a Rod1V/PPxY-less triple mutant was slightly more effective in stimulating recovery than a Rod1PPxY-less mutant (Figure 3.8A), although this difference could argueable be due to the difference in their level of expression (Figure 3.8B). Nonetheless, it is clear that the VPSY site contributes to Rsp5 binding to Rod1 in vitro, as judged by GST-pull down measurements (Figure 3.8C). Thus, the adaptation-promoting ability of Rod12A, Rod16A or Rod18A does not require intact V/PPxY motifs in these proteins.

However, another possibility to explain the potency of such mutants is that lack of phosphorylation allows these α-arrestin variants to form homo-oligomers with endogenous Rod1 (or hetero-oligomers with Rog3/Art7, or 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, Rod18A and Rod18A V/PPxY-less were equally efficacious in promoting recovery from pheromone-induced growth arrest (Figure 3.7C) and were expressed at an equivalent level (Figure 3.7D). Thus, Rod18A 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, as we observed before for its paralog Rog3 (Alvaro et al., 2014).
Figure 3.7 Hyperactive Rod1 does not require Rsp5 binding to regulate the mating pathway. (A) Adaptation-post pheromone as described in Figure 3.1A with versions of Rod1 in which all three Rsp-5 binding sites (V/PPxY) are mutated. (B) Confirmation of α-arrestin expression as described in Figure 3.2C. (C) Adaptation-post pheromone as described in Figure 3.1A with Rsp-5 binding sites (V/PPxY) mutants in cells lacking endogenous Rod1, Rog3 and Ldb19. (D) Confirmation of α-arrestin expression as described in Figure 3.2C.
Figure 3.8 Rod1 can function independently of all three PY motifs. (A) Adaptation-post pheromone as described in Figure 3.1A. (B) Confirmation of a-arrestin expression as described in Figure 3.2C. (C) Cultures of a GEV derivative of the protease deficient strain BJ5459 expressing the indicated mutant of Rod1 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.
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, indicating that, normally, the actions of these α-arrestins contribute to Rsp5-mediated ubiquitylation-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 ubiquitylation (Terrell et al., 1998; Hicke et al., 1998; Toshima et al., 2009) and required for its clathrin-mediated endocytosis (Ballon et al., 2006; Dores et al., 2010). Likewise, truncations of Ste2 that remove its entire C-terminal cytosolic tail such as Ste2(Δ296-431), 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 Rod 8A V/PPxY-less to promote adaptation quite potently (Figure 3.7A), suggested that, in the absence of phosphorylation, a desensitization mechanism distinct from endocytosis was occurring.

As one means to address this issue, we asked whether the Rod1 6A 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(Δ296-431) were the sole source of this receptor. Indeed, we found that Rod1 6A V/PPxY-less was able to stimulate recovery even more efficiently in cells expressing Ste2(7K-to-R) and Ste2(Δ296-431) than in cells expressing wild-type Ste2, and to do so more effectively than wild-type Rod1 (Figure 3.9A). Similar to what we observed before in cells expressing wild-type Ste2 (Alvaro et al., 2004), both Rog3 and a Rog3 truncation mutant that removes all three of its V/PPxY motifs also effectively promoted recovery in cells expressing Ste2(7K-to-R) and Ste2(Δ296-431) as the sole source of this receptor (Figure 3.9A). Although there were differences in the level of expression of these proteins that may contribute to their observed phenotypes (Figure 3.9B), we do not think that they are sufficient to explain their relative efficacy in promoting adaptation. In any event, it is clear that, in the absence of its phosphorylation, Rod1 promotes 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 -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, compared to either rod1Δ rod3Δ cells or Ste2(7K-to-R) cells. Indeed, as judged by the halo bioassay, we observed an additive effect of combining a rod1Δ rog3Δ double mutation with the Ste2(7K-to-R) mutation (Figure 3.9C) that was both reproducible and statistically significant (Figure 3.9D).

The fact that, in the absence of its phosphorylation, Rod1 can still promote adaptation independently of Rsp5-mediated receptor ubiquitylation 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), derivatives of Rod1 that are largely unphosphorylated and unable to associate with Rsp5 still promote efficient adaptation (Figure 3.8D and E), indicating a third mechanism by which this α-arrestin promotes desensitization of the pheromone-response pathway.
Figure 3.9 Rod1 and Rog3 mediate adaptation independently of Ste2 ubiquitinylation. (A) Adaptation-post pheromone as described in Figure 3.1A in cells where the endogenous gene of STE2 was replaced by Ste27KR or Ste2Δ296. (B) Confirmation of α-arrestin expression as described in Figure 3.2C. (C) Pheromone sensitivity of a Ste2-mcherry bar1Δ (or Ste27KR-mcherry bar1Δ) strain and otherwise isogenic rod1Δ rog3Δ Ste2-mcherry (or Ste27KR-mcherry) bar1Δ strain with 600ng α-factor spotted on filter disc, plated on SC-Ura. Plates were incubated for 2 days at 30°C (D) Quantification and statistical analysis of the change in halo diameter (n = 6). The average halo diameter for control cells was set at 100% and the halo sizes for each mutant were normalized to the control. Error bars, ± SEM; * p <0.001. (E) Adaptation-post pheromone as described in Figure 3.1A in cells lacking the formin Bni1.
DISCUSSION

Because endocytosis of many integral PM proteins in yeast is regulated by one or more of its 14 identified \(\alpha\)-arrestins (Lin et al., 2008; Nikko et al., 2008; Becuwe et al., 2012; O'Donnell et al., 2010; O'Donnell et al. 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 \(\alpha\)-arrestin is recruited to a particular target. Recent studies demonstrate that phosphorylation of an \(\alpha\)-arrestin either inhibits its ability to stimulate internalization of its target (Shinoda & Kikuchi, 2007; Lin et al., 2008; Nikko et al., 2008; MacGurn et al., 2011; Becuwe et al., 2012; Merhi & Andre, 2012; O'Donnell et al., 2013) or causes the \(\alpha\)-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 \(\alpha\)-arrestin to promote adaptation in the mating pheromone response pathway, where its apparent target is the \(\alpha\)-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 Als) 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.

Unexpectedly, 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 sites that are phosphorylated by Snf1 both \textit{in vivo} and \textit{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; Hedbäcker & 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<sup>6A</sup>, 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; 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<sup>6A</sup> 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); hence, it is still possible that certain AMPKLs redundantly phosphorylate Rod1 at its Snf1 sites when cell 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 the Target Of Rapamycin (TOR) Complex (TORC2) (Roelants et al., 2010; Roelants et al., 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<sup>6A</sup> 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 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 either in a cell cycle-dependent manner and/or subject to feedback phosphorylation by Fus3, the MAPK specifically activated by mating pheromone response pathway (Hao et al., 2013; 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 Ca<sup>2+</sup> (Ohsumi & 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 Ca\(^{2+}\) 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 3.10) in which Rod1 has at least two distinct mechanisms for blocking the function of Ste2 and thus preventing 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, engagement of the clathrin-dependent endocytosis machinery, followed by internalization and destruction of Ste2 in the vacuole (Alvaro et al., 2014). However, 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 more facilely adopt a conformation much like that found in \(\beta\)-arrestin (Shukla et al., 2014) and 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 \(\alpha\)-arrestins, the N- and C-lobes of their arrestin folds undergo a dramatic rotation with respect to one another in order to engage their target receptors (rhodopsin and \(\beta2\)-adrenergic receptor, respectfully) (Kang et al., 2015a). Thereby, visual arrestin and \(\beta\)-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 and this binding is mutually exclusive with occupancy of these receptors by their cognate G-proteins (Attramadal et al., 1992; Craft et al., 1994; Lohse et al., 1992). Indeed, consistent with this same kind of role for 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 \(\alpha\)-arrestins can promote a Rho1- and formin-requiring, but clathrin-independent, mechanism for internalization of certain integral PM proteins (Prosser et al., 2011; Prosser et al., 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.
Figure 3.10 Model for Rod1 phospho-regulation. Under normal growth conditions, Rod1 binds Rsp5. This drives ubiquitin-dependent internalization of the GPCR Ste2. When active either Snf1 (low glucose) or Ypk1 phosphorylates and inhibits Rod1’s ability to downregulate the mating pathway. When Snf1- or Ypk1-dependent phosphorylation blocked, Rod1 can regulate the mating pathway in an Rsp5-independent manner. The exact mechanism or new binding partners remain unknown. Calcineruin (CN) dephosphorylates Rod1 at the specific Snf1 and Ypk1 sites, keep Rod1 in its active state.
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 may bind very tightly to Ste2, it is possible that a primary and physiologically relevant role 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 Ca\textsuperscript{2+} 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 \(\alpha\)-arrestins Bul1 and Bul2 alters the way that 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 \(\alpha\)- and \(\beta\)-, 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 \(\alpha\)-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 \(\alpha\)-arrestins in GPCR down-regulation. Thus, \textit{S. cerevisiae} continues to serve as a useful model to explore \(\alpha\)-arrestin function and related mechanistic aspects of GPCR biology.

**ACKNOWLEDGEMENTS**

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### Table 1. Yeast strains used in this study.

<table>
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<tr>
<th>Strain</th>
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<th>Reference</th>
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<td>(Alvaro et al., 2014)</td>
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<tr>
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*Generated by site-directed mutagenesis with synthetic oligonucleotides containing the desired codon alterations (using wildtype sequence in pRS426 vectors as the template). DNA from the corresponding gene was amplified from genomic DNA by PCR and then cloned into pEGKG.*
CHAPTER 4: Perspectives and Future Directions

In part of my doctoral dissertation research, as described primarily in Chapter 2, I identified and characterized the function of three α-arrestins and their roles in regulating internalization of a GPCR, Ste2, in S. cerevisiae (Alvaro et al., 2014). My work was the first to show definitively that the GPCR class of integral PM proteins is subject to down-regulation by α-arrestins. Because the S. cerevisiae genome lacks any genes encoding β-arrestin-like proteins, my work was also the first to show that α-arrestins are necessary and sufficient to promote endocytosis of a GPCR, despite somewhat conflicting evidence in mammalian cells that both β-arrestins and α-arrestins fulfill this role (Benovic et al. 1987; Lohse et al. 1990; Attramadal et al. 1992; Puca et al. 2013). Thus, my work provided new information about α-arrestin-cargo selectivity and also opened the door to investigate various other mechanistic aspects of α-arrestin function using yeast cells as a simple model organism.

As further described primarily in Chapter 3, my thesis research has also demonstrated that the function of Rod1/Art4, one of the α-arrestins involved in Ste2 down-regulation, is regulated in a complex manner by phosphorylation at specific sites by multiple protein kinases and by dephosphorylation by CN, a Ca\(^{2+}\)/calmodulin-activated phosphoprotein phosphatase (Alvaro et al., 2014). An emerging aspect (and seemingly general feature) of α-arrestins is that these proteins are subject to extensive post-translational modifications that affect or alter their functions (Becuwe et al. 2012; Jee et al. 2012; Merhi & Andre 2012; Wu et al. 2013; O'Donnell et al. 2013; Crapeau et al. 2014; O'Donnell et al. 2015). In conjunction with these other studies, my findings on phospho-regulation of Rod1 provide new insights about how α-arrestin function is differentially regulated in response to specific stimuli. The material that follows in this final chapter is a mixture of intriguing preliminary data and ideas that could be pursued in future experiments.

Rsp5-independent function

Although, normally, both Rod1 and Rog3 bind Rsp5 (and themselves substrates for this E3), one unexpected result from my work is that, under the right circumstances, both Rod1 and Rog3 can promote adaptation, as judged by recovery from pheromone-induced growth arrest, in an Rsp5-independent manner. When overexpressed, a truncated version of Rog3 that lacks all three of its Rsp5-binding sites stimulates desensitization very potently (Alvaro et al., 2014). Similarly, when overexpressed, mutants of Rod1 that cannot be phosphorylated by either Ypk1 or Snf1 and lack all three of its Rsp5-binding sites (such as Rod1\(^{8A \ V/PPxY}\)) also robustly promote desensitization. Moreover, both truncated Rog3 and unphosphorylated V/PPxY-less Rod1 are able to promote recovery in cells where the sole source of Ste2 is a version of this receptor that cannot be ubiquitinylated, either Ste2(7K-to-R) or Ste2(\(\Delta\text{296-431}\)) and, thus, cannot be internalized by clathrin-mediated endocytosis. I have three hypotheses to explain these observations.

The first is that both mutant α-arrestins behave in a β-arrestin-like manner, i.e. they bind via their arrestin fold directly to the intracellular domains of Ste2, thereby occluding interaction with and preventing further activation of the Gpa1(G\(\alpha\))-Ste4(G\(\beta\))-Ste18(G\(\gamma\)) heterotrimeric G-protein. This model fits all of the available data. The second idea is that both mutant α-arrestins are now biased to act via a recently described clathrin-
independent pathway for internalization of PM proteins (Prosser et al., 2011; Prosser et al., 2015). However, I have ruled out this possibility because both mutant α-arrestins are able to promote recovery in cells lacking a component (the formin Bni1) required for the clathrin-independent route just as well as they do in wild-type cells. A third, alternative that is difficult to rule out is that both of the altered α-arrestins are neomorphic mutants that have lost the capacity to associate with Ste2 and somehow acquired the capacity to interact with and block the function of another component of the mating pheromone response pathway and thereby squelch pathway signaling.

Both biochemical (Lohse et al. 1990; Attramadal et al. 1992; Craft et al. 1994) and recent structural analysis (Shukla et al., 2014) of β-arrestin and its association with the GPCR β2-AR, as well as structural analysis of the interaction of visual arrestin with its target GPCR rhodopsin (Kang et al., 2015a; Kang et al., 2015b), show that both proteins use their arrestin fold domain to bind tightly to the intracellular structural elements of both receptors and in manner that is mutually exclusive with receptor-G protein coupling. In particular, visual arrestin binds tightly to rhodopsin that has undergone a conformational change as the result of its light-induced phosphorylation. This binding is extremely rapid post light-activation and blocks downstream signaling in an internalization-independent manner (Doan 2006; Hanson et al. 2007). Consistent with this mode of action for Rod1, one of my as yet unpublished observations is that, when overexpressed, Rod1 is more potent in stimulating the recovery of cells that lack the receptor-binding RGS protein Sst2 (Ballon et al., 2006) than in cells where Sst2 is present. If Rod1 must interact with Ste2 to promote adaptation, and Sst2 binding to the receptor prevents access of Rod1 to its binding site, when Sst2 is absent, Rod1 access will no longer be impeded. In this same regard, I found that Rog3 promotes adaptation equally well in wild-type and sst2Δ cells. So, if the model for Rod1 is true, then either Rog3 outcompetes Sst2 because it has a much higher affinity for binding to Ste2 than Rod1 or that it binds to a different set of determinants on Ste2 than Rod1 does (and these contacts are not occluded by Sst2 association with the receptor). It will be interesting to test directly whether the presence of Sst2 is or is not able to block the recovery-promoting activities of Rod1^{2A V/PPxY-less}, Rod1^{6A V/PPxY-less} and Rod1^{8A V/PPxY-less}.

In addition to their intimate association with their cognate receptors, mammalian β-arrestins also act as scaffolds that recruit two classes of MAPKs (ERK and JNK) (Luttrell et al. 1999; DeFea et al. 2000; McDonald et al. 2000; Luttrell et al. 2001; Ahn et al. 2004). Thus, β-arrestins play a role in dampening the initial G-protein evoked signal while simultaneously stimulating alternative downstream signaling pathways at the PM (Tohgo et al. 2003). There are also claims that β-arrestins bind and regulate clathrin (Goodman et al., 1996) and other classes of signaling molecules, like calmodulin (Wu et al. 2006). Thus, it is formally possible and may not be too far-fetched to consider that Rod1 and/or Rog3 might have binding partners other than Ste2 that, in this case, contribute to a blockade of pheromone signaling after the initial response rather than initiating alternative signaling pathways.

As an aside, because our adaptation assay relies on overexpression, it is also formally possible that, in addition to direct regulation of its primary target, because the α-arrestins are overproduced they could have unintended negative effects on secondary targets not normally affected. As just one purely hypothetical example, if in addition to strong interaction with Ste2, Rod1 and/or Rog3 also weakly bound another protein
required for propagation of the mating pheromone signal, such as the MAPK Fus3, it is theoretically possible the \( \alpha \)-arrestins could titrate out the available pool of activated Fus3, thereby diverting it from efficiently phosphorylating its normal targets, or inhibiting its activation by preventing its binding to the scaffold protein Ste5 or association with its upstream activating kinases, the MAPKK Ste7. Despite this potential caveat of the use of an overexpression assay, we believe it is providing reliable information because the observed phenotypes are, as expected, the opposite of loss of function mutations in the same proteins. Specifically, a \( \text{rod}1\Delta \text{rog}3\Delta \) mutant displays a reproducible increase in sensitivity to pheromone-induced growth arrest (Alvaro et al., 2014). Furthermore, overexpression can sometimes be exploited to help confirm known interaction partners, as well as to help identify suspected or novel ones.

To provide insight into the mechanism of the potent Rsp5-independent mating pheromone response pathway down-regulation exerted by mutant \( \alpha \)-arrestins, like Rog3(\( \Delta 400 \)) and Rod1\(^{8A} \text{V/PPxY} \), future studies could employ the tripartite split-GFP system (Cabantous et al. 2013). In brief, in this approach, strand \( \beta 10 \) of the eleven-stranded \( \beta \)-barrel of GFP would be attached via a flexible linker to the N-terminal end of the \( \alpha \)-arrestin and co-expressed with a genomic library wherein every open-reading-frame in the \textit{S. cerevisiae} genome has been fused at its C-terminal end via a flexible linker to the \( \beta 11 \) strand of GFP, along with the incomplete nine-stranded GFP(\( \beta 1-9 \)), which is non-fluorescent. Intimate interaction between the \( \beta 10 \)-tagged \( \alpha \)-arrestin and a \textit{bona fide} \( \beta 11 \)-tagged partner allows for capture of GFP(\( \beta 1-9 \)), reconstitution of the complete \( \beta \)-barrel, and fluorophore formation. By contrast, if the two tagged proteins do not interact, GFP will not be reconstituted, and there will be no fluorescent signal (Figure 4.1A). Prior imaging studies have had difficulty detecting co-localization between \( \alpha \)-arrestins and their demonstrated PM targets, and I have found the same to be true in my own hands (unpublished results). Hence, another virtue of the tripartite split-GFP system is that, once formed, an interaction that is normally dynamic gets "locked" in place via the stabilization afforded by the reformation of GFP from its component parts. As I argued in Chapter 3, one way to view the phosphorylation of an \( \alpha \)-arrestin is to promote its active dissociation from its targets, and the same argument could be made for the observed Rsp5-dependent auto-ubiquitinylation of \( \alpha \)-arrestins. Hence, use of an \( \alpha \)-arrestin derivative like Rod1\(^{8A} \text{V/PPxY} \) in the tripartite split-GFP system, or in other imaging approaches, would likely give the best chance for visualizing its association with its targets. In this same regard, just as visual arrestin only binds tightly to the light-induced phosphorylated form of rhodopsin, it is likely that, if Rod1 associates in a similar way via its arrestin fold with Ste2, that this interaction will only occur for the \( \alpha \)-factor-bound form of the receptor. Hence, to be properly controlled, any experiments to visualize potential interaction of Rod1\(^{8A} \text{V/PPxY} \) with the receptor at the PM should be done in both wild-type and \( \text{ste}2\Delta \) cells in both the absence and presence of a saturating concentration of pheromone in cells (\( \text{bar}1\Delta \)) that lack the extracellular protease responsible for destruction of exogenously supplied \( \alpha \)-factor. In addition, we can use cells expressing Ste2\(^{7KR} \) as the sole source of Ste2, this mutant is trapped at the PM and may enhance Rod1 recruitment. To further enforce persistent residence of the receptor at the PM, one might also consider adding latrunculin-A to
Figure 4.1 Tripartite split-GFP to identify interaction partners. A. Model for using tripartite split-GFP to determine which components of the mating pathway Rod1 interacts with. Cells expressing Rod1-GFP11, Ste2^{7KR}-GFP10, and the GFP 1-9 core will show fluorescence if Rod1 interacts with the receptor at the membrane. Inset represents the possibility that Rod1 interacts with another component of the mating pathway. B. Using split-GFP to determine the circumstances for preferential binding of PM proteins. Rod1 may bind different PM targets under different conditions. For example, when exposed to pheromone, Rod1 may preferentially bind Ste2, therefore we would only see a fluorescent signal when Ste2 is tagged and not Jen1. The reciprocal might be true for cells grown under high glucose.
block actin filament formation (Ayscough, 2005) because both clathrin-mediated endosome formation and internalization and the clathrin-independent pathway both require the polymerization of actin filaments. In any event, a good control for testing the tripartite split-GFP approach might be the association of Rod1 with Rsp5— a signal should be observed between Rsp5 and wild-type Rod1, and not Rod1\(^{\Delta PP\alpha Y}\), and, conversely, a signal should be observed between Rod1 and wild-type Rsp5, but not Rsp5 in which its three WW domains have been mutated (Watanabe et al., 2015). Of course, unbiased use of this tripartite split-GFP system might reveal novel interaction partners for Rod1.

Another approach to discriminate at what level in the mating pheromone response pathway Rog3(\(\Delta 400\)) and Rod1\(^{\Delta 8A PP\alpha Y}\) are acting would be to use mutants that stimulate the pathway in a manner that does not depend on receptor-initiated events. Indeed, there are available constitutively-active versions of the MAPKKK Ste11, which when expressed from the \(\text{GAL}\) promoter, elicit mating pathway outputs in a pheromone-independent manner (Stevenson et al. 1992). If such an allele of Ste11 is able to stimulate the mating pathway even in the presence of overexpressed Rog3(\(\Delta 400\)) and Rod1\(^{\Delta 8A PP\alpha Y}\), then these \(\alpha\)-arrestins act only upstream, e.g. at the level of the receptor, whereas if overexpressed Rog3(\(\Delta 400\)) and Rod1\(^{\Delta 8A PP\alpha Y}\) block the effects of constitutively-active Ste11, then these \(\alpha\)-arrestins may act on a component downstream in the pathway, e.g. at the level of the MAPK Fus3, perhaps.

**Mechanism of phosho-regulation**

Although in my studies I was able to pinpoint specific residues in Rod1 that are phosphorylated by both Snf1 and Ypk1, I have yet to determine, at the mechanistic level, how this phosphorylation inhibits the ability of Rod1 to promote recovery from pheromone-induced growth arrest. Phosphorylation could interfere with the binding of Rsp5 to Rod1 (or vice-versa) (Figure 4.2); one way to explore that possibility would be to ask whether or not the phosho-mimetic mutant GST-Rod1\(^{\Delta E}\) pulls down much less Rsp5 than the unphosphorylatable mutant GST-Rod1\(^{\Delta A}\). Another possibility is that phosphorylation prevents the arrestin fold of Rod1 from binding to Ste2, either sterically or electrostatically, or by preventing a necessary conformational change. One way to address that possibility would be to use co-immunoprecipitation and compare the amount of FLAG-tagged Rod1\(^{\Delta A}\) versus Rod1\(^{\Delta E}\) that can be cross-linked to Ste2-mCherry in cells exposed to \(\alpha\)-factor and treated with latrunculin-A, for the reasons already discussed above. Another possibility is that phosphorylation generates phospho-epitopes on Rod1 that permit its binding to 14-3-3 proteins, as has been reported for certain other \(\alpha\)-arrestins (Merhi & Andre, 2012), thus sequestering Rod1 in the cytosol and thereby limiting the pool available for interaction with Ste2 at the PM. Indeed, when cells are grown in a non-fermentable carbon source, Rod1 is phosphorylated by Snf1 and it has been reported that this hyper-phosphorylated form of Rod1 is bound to the yeast 14-3-3 proteins Bmh1 and Bmh2 (Becuwe et al. 2012). Moreover, in the study cited, the authors argued that the 14-3-3 proteins occlude Rod1 from localizing to the PM and interacting with the lactate permease Jen1. However, Rod1 still interacted to a detectable degree with Bmh2 when cells were grown on glucose medium, a condition under which Rod1 actively stimulates endocytosis of Jen1. Therefore, there seems to be a need for more nuance in the model proposed.
Nonetheless, it is possible that, when phosphorylated specifically by Snf1, epitopes are generated on Rod1 that are preferred 14-3-3-binding motifs and that 14-3-3 binding occludes Rod1-cargo interactions. This scenario may certainly be occurring when cells are grown on galactose and Snf1 activity is high, where I observed that Rod1 is unable to promote adaptation (Figure 4.2). However, phosphorylation of Rod1 by Ypk1 and/or other protein kinases likely do not generate phospho-epitopes that are 14-3-3 binding sites and, thus, other mechanisms, such as some of the other alternatives already discussed above are needed to explain how phosphorylation negatively regulate the ability of Rod1 to promote adaptation. In this regard, in a recent study (Crapeau et al. 2014), it was shown that phosphorylation and 14-3-3 binding to the α-arrestins Bul1 and Bul2 and Aly1 and Aly2 switch the way these proteins bind to and regulate the general amino acid permease Gap1. Thus, phosphorylation may not only negatively regulate the function of an α-arrestin, but may also change its function and/or its cargo selectivity, thereby allowing the same limited repertoire of 14 α-arrestins to be preferentially recruited under the right circumstances to specific ones of the multitudinous classes of pumps, permeases, channels, transporters and receptors in the PM.
Under normal glucose conditions, Rod1 seems to have two mechanisms for regulating the mating pathway. The first is by recruiting Rsp5 to Ste2 and mediating internalization. The second mechanism we are less clear on, however we know it is independent of Rsp5. The switch in these mechanisms may be controlled through phosphorylation (by Ypk1 and other unidentified kinases). Under low glucose conditions, Rod1 is hyper-phosphorylated by Snf1 and binds 14-3-3 proteins. This prevents Rod1 regulation of the mating pathway.

**Figure 4.2 Model for phosphor-regulation of Rod1.** Under normal glucose conditions Rod1 seems to have two mechanisms for regulating the mating pathway. The first is by recruiting Rsp5 to Ste2 and mediating internalization. The second mechanism we are less clear on, however we know it is independent of Rsp5. The switch in these mechanisms may be controlled through phosphorylation (by Ypk1 and other unidentified kinases). Under low glucose conditions, Rod1 is hyper-phosphorylated by Snf1 and binds 14-3-3 proteins. This prevents Rod1 regulation of the mating pathway.
Phosphorylation sites in Rod1: Snf1
In Chapter 3, I demonstrated that Rod1 is phosphorylated by the protein kinase Snf1 both in vitro and in vivo. However, several pieces of data indicated that Rod1 is also phosphorylated by other protein kinases and at additional sites. First, I found that, even in cells lacking Snf1, wild-type Rod1 is not as active in promoting recovery as Rod 6A (lacking all known Snf1 sites). Additionally, Rod 16A is hyperactive in promoting recovery even on glucose, where Snf1 activity is low. Furthermore, despite the fact that Rod 16A already lacks phosphorylation at all of its Snf1 sites, its potency in promoting adaptation is lost almost completely in CN-deficient cells, indicating that phosphorylation(s) at another position(s) also needs to be removed to allow Rod1 to function.

The consensus phospho-acceptor motif I used to identify putative Snf1 sites was $\Phi xR/KxxSxxx\Phi$ (Hardie DG et al., 1998; Mok et al. 2010). However, this motif contains within it shorter sequences that fit the consensus sites phosphorylated by some of the AMPKs, such as Gin4 (RxxS) and Kcc4 (RxxS) (Mok et al. 2010), as well as other types of protein kinases, such as Rck2 (R/KxxS), Skm1 (RxxS), and Vhs1 (ΦxRxxS) (Mok et al. 2010), to name a few potential examples. Therefore, it is possible that one or more of the Snf1 sites I predicted are being phosphorylated by yet other protein kinases.

It has been reported that overexpression of protein kinase Prr1 inhibits pheromone-induced signaling and does so downstream of Fus3 MAPK, possibly at the level of the transcription Ste12 (Burchett et al. 2001). Two of our deduced Snf1 sites (S447 and S706) match moderately well the consensus motif for Prr1 (RSSS) (Mok et al. 2010). Hence, it is possible that Prr1 may phosphorylate these sites. However, my work has shown that phosphorylation at these sites interferes with Rod1 function in promoting adaptation; so, if Prr1 did phosphorylate and block Rod1 function, it would act against its other purported role in squelching pheromone pathway signaling.

Yak1 is a protein kinase that mainly phosphorylates targets in the nucleus. PKA-mediated phosphorylation of Yak1 prevents its nuclear entry. Thus, in the presence of glucose, where cAMP generation is robust and PKA is highly active, Yak1 is phosphorylated, excluded from the nucleus, and kept inactive by binding to 14-3-3 proteins (Moriya et al. 2001; Lee et al., 2011). Upon glucose starvation, PKA activity is low, Yak1 enters the nucleus and phosphorylates transcription factors that limit growth by, for example, by inhibiting the expression of ribosomal protein genes (Lee et al., 2011). Thus, it is formally possible that Yak1 is able to phosphorylate Rod1 when glucose is low, such as on galactose medium. However, it seems unlikely to do so because it is located in the nucleus when glucose is limiting, whereas Rod1 is a cytosolic protein. Moreover, the consensus motif for Yak1 (RxxS/Tp) (Mok et al. 2010) contains a Pro at position -1, which is a residue not present at this location in any of the six Snf1 sites in Rod1.

In yeast, the atypical protein kinase Target of Rapamycin Complex 1 (TORC1) is active when the amino acid supply is abundant and is inactive when amino acids become limiting (Loewith & Hall, 2011). TORC1 exerts global control on many aspects of cell metabolism, on the one hand, by phosphorylating and activating certain downstream protein kinases that promote growth, such as Sch9 (Urban et al. 2007) and Pkc1, and, on the other hand, by phosphorylating and inactivating protein kinases whose function is only needed when nutrients become limiting, such as Npr1 and Atg1. It has been shown that, under starvation conditions, TORC1-mediated inhibition of Npr1
is alleviated and Npr1 thus activated phosphorylates and blocks the function of Ldb19/Art1, thereby helping to prevent endocytosis of certain nutrient permeases just when the cell needs them in the PM the most (MacGurn et al. 2011). Sch9, by contrast, is most active when cells need to make more mass, such as during mitotic growth or during developmental transitions, like mating, where new proteins need to be made. Thus, it is an intriguing possibility that Sch9, whose consensus motif is \textit{RRΦS} (Mok et al. 2010), might act to enhance mating efficiency by phosphorylating Rod1 and one or more of the Snf1 sites, thereby allowing \textit{MATa} cells to retain more Ste2 in the PM.

**Phosphorylation sites in Rod1: Ypk1**

I was able to show that the reason Rod1\(^{6A}\), which lacks phosphorylation at all six of its Snf1 sites, is inactive in CN-deficient cells was due primarily to inhibitory phosphorylation at two other sites, which I demonstrated are sites for the protein kinase Ypk1 both \textit{in vitro} and \textit{in vivo}. Remarkably, on glucose medium, a mutant lacking just these two sites, Rod1\(^{2A}\), displayed a adaptation-promoting activity that was greater than Rod1\(^{6A}\). Moreover, removal of the two Ypk1 sites from Rod1\(^{6A}\) generated a mutant, Rod1\(^{8A}\), that was even more efficacious.

On the basis of electrophoretic mobility in SDS-PAGE, it is clear that the majority of the phosphorylation that occurs on Rod1 is at its Snf1 and Ypk1 sites. Wild-type Rod1 runs as a broad diffuse smear indicative of multiple isoforms that are collapsed to a single major faster mobility species upon CIP treatment. By contrast, the Rod1\(^{6A}\) mutant already runs as a major prominent band in the absence of CIP treatment; yet, this species is still trailed by a minor smear of slower mobility isoforms that are eliminated upon CIP treatment. This behavior indicates that Rod1 is phosphorylated at yet other sites. This conclusion is in agreement with genome-wide phosphoproteomic analyses (Ficarro et al. 2002; Swaney et al. 2013) that have detected phosphorylation \textit{in vivo} at many residues in Rod1 other than the Snf1 and Ypk1 site I mutated (Figure 4.3A). Which kinases are phosphorylating these additional sites and what effect they may have on Rod1 function is yet to be determined.

In this regard, a particularly strong candidate for phosphorylation and regulation of Rod1 is Fus3, the terminal MAPK of the mating pheromone response pathway (Madhani et al. 1999; Roberts et al. 2000; Slessareva & Dohlman 2006). Like all MAPKs, Fus3 phosphorylates only at S/TP sites (Mok et al. 2010) in its target proteins. The sequence of Rod1 contains six potential MAPK sites (Figure 4.3B), at four of which phosphorylation \textit{in vivo} has been detected by phosphoproteomics (Swaney et al. 2013). Five of the six putative Fus3 sites are in the C-terminal domain of Rod1 (Figure 4.3C). Thus, it is possible that, after its activation by the mating pheromone response pathway, Fus3 could phosphorylate Rod1. Given that CN-mediated dephosphorylation of Rod1 is required for its ability to down-regulate Ste2 late in the mating response to ensure complete clearance of this now unnecessary protein, perhaps Fus3-mediated phosphorylation helps impose a temporal delay in the dephosphorylation of Rod1 until just the right time. Alternatively, since addition of pheromone also seems to stimulate Ste2 internalization quite rapidly (Chvatchko et al. 1986; Jenness & Spatrick 1986; Zanolari & Riezman 1991; Davis et al. 1993; Schandel & Jenness 1994), then, if Rod1 really is a target for Fus3, perhaps phosphorylation at these S/TP sites somehow stimulates the ability of Rod1 to promote Ste2 endocytosis.
**A**

1  MFSSSRPSK  EPLLFDIRDIE  NLDNVILIK  GPPDEASSVL  LSGTIVLSTIT
51  EPIIQIKSLAL  RLFGRLRNI  PTVLQTVHIP  HKKSKFERN  IYSHFWDNFN
101  IKYFPQNYD  NHHNKKTIG  SKSTSNALAL  PKRRALETA  SLISSNQSTS
151  ASKNHVLTVK  CNPFQPSFIS  IPGSLVESVE  GLPNAAVTYA  LEATIEPRPQ
201  PDLICKHKLRL  VIKIRLADEV  ELSETVSDVBN  SWPKEVYTI  SIPTKAIAIG
251  SSTMINILIV  PILGGLKGIP  VRISLLVESQ  YCGSYGGVIN  QERMVAKKLK
301  KDPLKHVAQI  KKKRSLNNEA  DEGVDTDFGE  FQDKWEVRAL  LNIAPASLTKC
351  SQDCRILSNI  KVRRHIIKPTI  SLLNPDPGHS  ELRAALPVQL  FSFPVVPNVN
401  KGSVFIERTL  KTFGPSYQVT  SQDNSFNSSK  NFVDFDDEDV  IFORSASALQ
451  LSSMPTIVSG  STLNINSTDA  DTAHAVADTM  VTSLMVPPNY  GHHYVDRYVG
501  EVTNEDEGA  SAGGSAVEDQ  AINHHQNYLH  GDGNNRNFN  LAPNQIKIIE
551  DGSMLCDDR  GDEVQVSNNL  LVBLSNLTIE  NWNNSNSAN  RYNININAGL
601  NSPSLPSFA  HLRANNSYR  QSSTSTSLKD  LELDSLREVE  SDDXAMKSDM
651  IGDDLPPKYP  EEEVQRQQEB  KIBLERFQIL  HKKSLSLFP  LGPSKSSSN
701  LRKSSRSHL  SHSPRPRNS  GSVNLQQLA  RNRNTSSFLN  NLSFTSAKSS
751  TGSRSHPFNM  TTSTNSNSSS  KNNSHFDRTD  STSDANKPRED  EENYTSATHN
801  RRSRSSVRS  NNNNSFLQPG  TGFSAANLMEM  FTKDRDS*

**B**

1  MFSSSRPSK  EPLLFDIRDIE  NLDNVILIK  GPPDEASSVL  LSGTIVLSTIT
51  EPIIQIKSLAL  RLFGRLRNI  PTVLQTVHIP  HKKSKFERN  IYSHFWDNFN
101  IKYFPQNYD  NHHNKKTIG  SKSTSNALAL  PKRRALETA  SLISSNQSTS
151  ASKNHVLTVK  CNPFQPSFIS  IPGSLVESVE  GLPNAAVTYA  LEATIEPRPQ
201  PDLICKHKLRL  VIKIRLADEV  ELSETVSDVBN  SWPKEVYTI  SIPTKAIAIG
251  SSTMINILIV  PILGGLKGIP  VRISLLVESQ  YCGSYGGVIN  QERMVAKKLK
301  KDPLKHVAQI  KKKRSLNNEA  DEGVDTDFGE  FQDKWEVRAL  LNIAPASLTKC
351  SQDCRILSNI  KVRRHIIKPTI  SLLNPDPGHS  ELRAALPVQL  FSFPVVPNVN
401  KGSVFIERTL  KTFGPSYQVT  SQDNSFNSSK  NFVDFDDEDV  IFORSASALQ
451  LSSMPTIVSG  STLNINSTDA  DTAHAVADTM  VTSLMVPPNY  GHHYVDRYVG
501  EVTNEDEGA  SAGGSAVEDQ  AINHHQNYLH  GDGNNRNFN  LAPNQIKIIE
551  DGSMLCDDR  GDEVQVSNNL  LVBLSNLTIE  NWNNSNSAN  RYNININAGL
601  NSPSLPSFA  HLRANNSYR  QSSTSTSLKD  LELDSLREVE  SDDXAMKSDM
651  IGDDLPPKYP  EEEVQRQQEB  KIBLERFQIL  HKKSLSLFP  LGPSKSSSN
701  LRKSSRSHL  SHSPRPRNS  GSVNLQQLA  RNRNTSSFLN  NLSFTSAKSS
751  TGSRSHPFNM  TTSTNSNSSS  KNNSHFDRTD  STSDANKPRED  EENYTSATHN
801  RRSRSSVRS  NNNNSFLQPG  TGFSAANLMEM  FTKDRDS*

**C**

- **V/PPxy** motif
- **ΦxR/KxxSxxxΦ** - Snf1 sites
- **RxxSxxΦ** - Ypk1 sites
- **S/TP** - MAPK (Fus3) sites

- **Arrestin Fold**
- **V/PPxy Motif**
- **Snf1 site**
- **Ypk1 site**
- **MAPK site**
Figure 4.3 Additional phosphorylation sites in Rod1. A. Protein sequence of Rod1. All residues shown to be phosphorylated in vivo (from global studies (Ghaemmaghami et al. 2003; Swaney et al. 2013)) shown in blue. The sites that overlap with predicted sites we identified are bolded and circled. B. Protein sequence of Rod1, arrestin fold bolded and underlined. V/PPxY motifs shown in dark blue, Snf1 sites in green, Ypk1 sites in pink and putative MAPK sites in orange. C. Cartoon of Rod1p with annotated phospho-sites depicted in B.


**Input-specific regulation of Rod1**

Along these lines, it would seem important to study the kinetics of phosphorylation (and dephosphorylation) of Rod1 at all of its different sites before, immediately after, and following prolonged exposure to mating pheromone. At least the six Snf1 sites and the two Ypk1 sites we identified are clearly inhibitory to the function of Rod1 in stimulating Ste2 internalization and recovery from pheromone-induced growth arrest. It will be interesting to see whether phosphorylations at any other positions have a positive role and/or change the cargo preference of Rod1. Addressing these questions may help elucidate how a limited number of α-arrestins can mediate the endocytosis of such a large number of different types of PM proteins in response to particular stimuli.

Indeed, Rod1 has been shown to be required for the internalization of PM membrane proteins other than Ste2, including Jen1 (Becuwe et al. 2012), Hxt1 and Hxt3 (O'Donnell et al. 2015), and Hxt6 (Nikko & Pelham, 2009). It would be very informative to determine whether the Rod1 alleles that I have identified that are hyperactive for its function in the mating pathway are more (or less) active in promoting the endocytosis of these other cargoes. If phosphorylation at the Snf1 and Ypk1 sites has a general negative effect on Rod1 function, I would expect that hyperactive mutants, like Rod1\textsuperscript{8A}, will promote internalization of Jen1, Hxt1, etc. more efficiently than wild-type Rod1.

It has been reported that there is crosstalk between glucose sensing pathways and the mating response; more specifically, when cells are starved for glucose, the mating pathway is negatively regulated through Snf1-dependent phosphorylation of Gpa1 (Clement et al. 2013). However, given the results I have obtained in this thesis, upon glucose starvation, Snf1-mediated phosphorylation will also block the ability of Rod1 to promote Ste2 down-regulation. It would seem physiologically counter-productive to maintain the receptor in the PM, yet somehow reduce coupling to its cognate heterotrimeric G protein. Perhaps, under glucose-limiting conditions, it is more important to block Rod1-mediated endocytosis of a permease, like Jen1, for an alternative carbon source, like lactate, than it is to leave Ste2 in the PM. Alternatively, perhaps differential phosphorylation of Rod1 at particular residues affects its affinity for specific targets. If so, it would be the first evidence that phosphorylation of an α-arrestin can re-direct its function to different PM substrates.

As previously mentioned, the tripartite split-GFP system might be an extremely useful tool to address this question. For example, different PM proteins could be tagged on their C-terminus with β11 and expressed with N-terminally β10-tagged Rod1, along with GFP(β1-9). If the proteins only interact under certain conditions, a fluorescence signal should be stimulus-specific. Ste2 may only interact with Rod1 when pheromone is present, whereas interaction with another target, such as Jen1, may be prevented. The reciprocal experiment can also be done— on high glucose Rod1 should associate with Jen1, but likely not with Ste2 (Figure 4.1B).

**Interplay between phosphorylation and ubiquitinylation**

Previous studies have suggested an interplay between the phosphorylation state of Rod1 and its ubiquitinylation (Becuwe et al. 2012; O'Donnell et al. 2015). While it is unclear based on these studies how these two post-translational modifications affect
each other, it is clear that one modification may influence the other. These studies suggest that phosphorylation of Rod1 prevents ubiquitylation by Rsp5 because when cells are grown in galactose (where Snf1 activity is high), ubiquitylation is prevented despite that fact that Rod1 is bound to Rsp5 under this condition (Becuwe et al. 2012). The authors suggest that the 14-3-3 binding may occlude ubiquitylation sites in Rod1. When we analyze our Rod16A allele (lacking the Snf1 sites) by SDS-PAGE and immunoblotting, a complete collapse in the banding pattern occurs when the samples are treated with CIP (See Figure 3.4D). By contrast, for wild-type Rod1 and Rod12A (lacking the Ypk1 sites), a CIP-resistant band remains, indicating presence of another post-translational modification. If this modification is ubiquitylation, it could imply that phosphorylation at the Snf1 sites can indeed affect ubiquitylation of Rod1.

Aside from the possibility that binding of a 14-3-3 protein to a phospho-site on Rod1 may block Rod1 ubiquitylation by occluding access of Rsp5 to certain target Lys residue(s) or by occluding certain PPxY motifs thereby blocking Rsp5 binding at certain positions, it is also possible, as mentioned earlier, that phosphorylation of Rod1 at specific sites is, by itself, sufficient to mask a nearby Lys or prevent Rsp5 from docking on a PPxY site. One of the Snf1 sites I identified is S641 located in the VPSY motif (Figure 4.3B). Thus, this site may normally be phosphorylated preventing its interaction with Rsp5, which may explain why mutation of either of the PPxY motifs in Rod1 (PPNY to PANA or PPAY to PAAA), without changing VPSY, was sufficient to prevent overexpressed Rod1 from promoting adaptation (Alvaro et al., 2014). Hence, it would be interesting to compare the properties of Rod1PANA PAAA to Rod1PANA PAAA VPAY. Unlike VPSY, the VPAY motif may be able to restore significant Rsp5 binding and, hence, detectable function. Curiously, however, in at least one phosphoproteomic study (Swaney et al. 2013), Tyr642 was reportedly also phosphorylated in vivo, a very rare occurrence in yeast proteins that are not themselves protein kinases. Hence, if this modification persists even when the VPSY motif is mutated to VPAY, then it may be sufficient to diminish Rsp5 binding.

Mapping residues in Rog3Δ400 important for function
Many studies have begun to map determinants in the β-arrestins that are important for their functions (Lefkowitz 2005; Hanson et al. 2006; Gimenez et al. 2012; Lohse & Hoffmann 2013; Kang et al. 2013; Kang et al. 2015); however, relatively speaking, nothing is known about the elements in the arrestin fold of α-arrestins that mediate their interactions with their targets. Therefore, it would be useful to mutationaly map regions important for α-arrestin function. To begin to address this issue, I chose to focus on Rog3Δ400 because this truncation is about half the size of the full-length protein, is more stable than the full-length protein, includes just it predicted arrestin fold, yet is fully functional in terms of its ability to robustly promote recovery from pheromone-induced growth arrest (Alvaro et al., 2014). Hence, I chose to initiate a mutagenesis screen of Rog3Δ400. To assess function, I wanted to develop another method, aside from the halo bioassay, to monitor recovery from pheromone-induced growth arrest. I tried overexpressing Rog3Δ400 in cells grown in liquid culture and monitored growth rates by light scattering. Overexpression of GST-Rog3Δ400 in the absence of pheromone slightly inhibited cell growth when compared to the vector (GST only) control (Figure 4.4A, top left). When I treated the cells with six different pheromone concentrations and monitored
Figure 4.4 identifying regions of Rog3Δ400 important for function. A. Growth assay to access effect of overexpression of Rog3Δ400 on pheromone-induced growth arrest. Cells were grown in SC Ura- and treated with 20μM β-estradiol in mid-exponential log phase. After 3 hours of induction cells were treated with different concentrations of α-factor (0, 5nM, 50nM, 500nM and (5μM and 50μM, data not shown)) for 3 days. Growth was measured using infinite M1000 TECAN plate reader. Black line – overexpression of GST, green line – overexpression of GST-Rog3Δ400. B. Overexpression of either the N- or C- terminal domain of Rog3. Experiments were performed as previously described in Chapters 2 and 3. C. Summary of mutagenesis screen. Rog3Δ400 was modeled using the structural prediction program Phyre2.0 and analyzed using the Chimera software. Regions in yellow represent the regions that are dispensable for Rog3 function while regions in pink represent regions necessary for function. Regions in blue are those not yet covered in our analysis.
growth for three days, I was surprised to see that overexpression of Rog3∆400 did not confer a very dramatic rescue from growth arrest, except perhaps when the pheromone concentration was 500 nM (Figure 4.4A, bottom left). By comparison to the dramatic phenotypic differences seen in assays conducted agar plates, these results were disappointed and inexplicable. For this reason, I continued to use the halo bioassay on plates.

As mentioned, Rog3∆400 is truncated just after its predicted arrestin fold. The arrestin fold is composed of two discrete elements, an N-lobe and a C-lobe. Since each lobe folds as an independent unit, I tested whether either alone was sufficient for function. Using the program Phyre2.0, I mapped the N-arrestin fold as ending at residue 218, and cut the protein into two halves near this point. Upon overexpression, unlike Rog3∆400 itself, neither half alone was able to induce adaptation (Figure 4.4B), despite them being expressed at relatively equal levels (data not shown). This piece of data suggests that neither the N-lobe nor the C-lobe can function independently of the other; however, I did not try co-expressing these two fragments together to see if they could work in trans.

Next I decided to perform alanine scanning mutagenesis on Rog3∆400, systematically mutating blocks of eight residues to Ala and assaying for function. Using such a method, it is easiest to interpret those mutants that retained function because they indicate that the mutated block of eight residues is dispensable for function. Preliminary data identified several such regions in Rog3∆400. These regions are indicated in yellow in the structural model shown in Figure 4.4C. The regions in pink are the areas that when mutated rendered Rog3∆400 non-functional. Since many of these lie within β-sheets, and poly-Ala has a strong propensity to form an α-helix, it is possible that these alterations do not disrupt residues in the arrestin fold that contact Ste2, but rather merely interfere with proper folding of the arrestin domains and/or grossly alter their structure, stability, or solubility. To address this in the future, I would suggest making a fusion protein, such as Rog3∆400-His3 (fused to an enzyme responsible for survival in the absence of histidine), and continuing the mutagenesis with this construct. Only those mutants that remained folded and soluble, and were not degraded, aggregated or insoluble, would support growth on medium lacking histidine. Another way to improve the screen would be to model and predict surface-exposed regions in Rog3 and begin by mutating such residues individually, rather than in blocks of eight, because surface residues are more likely to be involved in protein-protein contacts and less likely to be important for folding of the Rog3 core and its stability. I have engaged the participation of a very able undergraduate, Ms. Rebecca Kuan, to assist me with these particular studies.

I could also use a biased approach and model the predicted arrestin fold in Rog3 on the crystal structures of visual arrestin and β-arrestin to try and pinpoint residues that are important for receptor binding (and binding to other partners). Several studies have identified specific regions and residues in β-arrestins that are important for interaction between the arrestin and its partner GPCR (Lefkowitz, 2005; Hanson et al. 2006; Wu et al. 2006; Gimenez et al. 2012; Lohse & Hoffmann 2013; Kang et al. 2013; Kang et al. 2015). One recent study identified seven specific residues in β-arrestin-3 that altered interactions with specific mammalian GPCRs (Gimenez et al. 2012). Earlier this year, the first crystal structure of a GPCR-arrestin complex was identified (Kang et al. 2015). The crystal structure reveals several contact regions between light-activated rhodopsin
and the visual arrestin. Using these studies as guides, I can attempt to model the structure of the arrestin fold of Rog3 and mutate analogous residues.

Regulation of other GPCRs in *S. cerevisiae*

Since my work was the first to characterize a role for any α-arrestin in a GPCR-initiated signaling pathway in yeast, an obvious question is whether the same α-arrestins I identified (Ldb19/Art, Rod1/Art4 and Rog3/Art7) that affect Ste2 are involved in mediating internalization of any other yeast GPCR, or whether others of the 14 yeast α-arrestins are involved. Is there a specific GPCR-arrestin code? In yeast, there are at least four GPCR-like PM proteins: Ste2, receptor for α-factor; Ste3, receptor for α-factor; Gpr1, a glucose and sucrose sensor; and, Rim21, a pH sensor. Recent work has shown that the paralogs Aly1 and Aly2, but not Rod1 and Rog3, are involved in both clathrin-mediated and clathrin-independent internalization of Ste3 (Prosser et al. 2015). Thus, Rod1 and Rog3 specifically regulate Ste2, whereas Aly1 and Aly2 specifically regulate Ste3 (Figure 4.5A). These data suggest there is a unique GPCR-arrestin code. Interestingly, although Sst2 binds both Ste2 and Ste3 (Ballon et al. 2006), it seems that the arrestins do not, suggesting that there are unique contact sites that distinguish how the RGS protein and the α-arrestins contact these receptors.

Despite the fact that hyperactive Rod1 and Rog3 can regulate the mating pathway in MATα cells expressing a tailless Ste2, Ste2Δ296, I thought that some specificity might be conferred through the C-terminal tails of the pheromone receptors. To test this hypothesis, I created a chimeric protein in which the C-terminal tail of Ste2 was replaced with the C-terminal tail of Ste3 (Ste2(1-308)-Ste3(289-470), referred to as Ste2-3tail). I asked if this swap was sufficient to change specificity of α-arrestin regulation (Figure 4.5A). Compared to wild-type Ste2, functionality of the Ste2-3tail chimera was markedly reduced, as judged by the halo size (Figure 4.5B). Nonetheless, recovery was promoted by Rod1 and Rog3, and not by Aly1 or Aly2, just as observed for wild-type Ste2 (Figure 4.5B). Thus, the C-terminal tail does not appear to be an important determinant of the specificity of the GPCR-arrestin interaction and, taken with the data shown in Chapter 3, further suggest that the C-terminal tail is neither necessary nor sufficient for α-arrestin binding.

Since the specificity for receptor recognition by an α-arrestin must presumably lie in elements accessible from the cytosol, I also generated a Ste2-Ste3 chimera in which all the intracellular loops were Ste3 sequence and all the external loops and transmembrane domains were from Ste2. However, this chimera yielded no detectable response to α-factor, suggesting that is likely improperly folded.
Figure 4.5 C-terminus of Ste2/3 does not determine arrestin-GPCR code. A. Summary of arrestin-GPCR code. Top panel - Ste2 is regulated by Rod1 and Rog3 (in addition to Ldb19) while Ste3 is regulated by Aly1 and Aly2 (in addition to Ldb19). Bottom panel – C-terminal tail swap of Ste2 and Ste3 may alter the arrestin code. B. Overexpression of the indicated arrestins in cells expressing wildtype Ste2 or the chimeric Ste2<sup>3tail</sup>, which replaces the C-terminal tail of Ste2 with Ste3. Experiments were performed as previously described in Chapters 2 and 3.
Figure 4.6 arrestin-GPCR code in *S. cerevisiae*. There are three GPCRs in *S. cerevisiae*: Gpr1 (black), Ste2 (dark purple) and Ste3 (light purple), Rim21 is not depicted in this cartoon. Rod1/Rog3 and Aly1/Aly2 regulate Ste2 and Ste3 respectively. However, the α-arrestins that regulate Gpr1 internalization have yet to be determined.
Although I was unable to gain more detailed insight using chimeric receptors, I do believe that there are specificity determinants in GPCR-α-arrestin interaction waiting to be elucidated. It would also be useful in this same regard to determine the α-arrestins responsible for internalization of Gpr1 and Rim21 (Figure 4.6). Lastly, my work and that of other yeast labs have delineated many of the PM targets of the 14 yeast α-arrestins. Since mammalian cells contain roughly 800 GPCRs, it would be exciting to extend this work to mammalian cells and determine which ARRDCs interact with which GPCRs and whether there are new ARRDCs that have not yet been identified on the basis of homology (or predicted structure). Perhaps, ectopically overexpressing each ARRDC in a high throughput manner and examining by mass spectrometry which PM proteins become the most depleted might be one approach that could be used to gain new insights. In any event, I think that my work in S. cerevisiae served as a useful model for dissecting new details about GPCR regulation that will apply to all eukaryotes and will continue to be built upon to expand our understanding of GPCRs and the control of their function.
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