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Role of G-protein Regulation of Formins during Gradient Tracking in Saccharomyces cerevisiae

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ROLE OF G-PROTEIN REGULATION OF FORMINS DURING GRADIENT TRACKING IN *SACCHAROMYCES CEREVISIAE*

by

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ABSTRACT

The yeast *Saccharomyces cerevisiae* uses a GPCR to direct the pheromone response pathway. Haploid yeast detect and respond to pheromone gradients produced by the opposite mating type to find a mating partner. At a high dose of pheromone, yeast will form a short, focused mating projection in order to mate with yeast that are close by. At lower doses of pheromone, the yeast form a broader projection which grows towards the source of pheromone. The pheromone is detected by a GPCR, Ste2, which activates the Gα, Gpa1, initiating the production of new gene products for mating, and polarizes the cytoskeleton in the direction of the source of the pheromone, facilitating gradient tracking. Cells expressing a mutant Gα are unable to track a gradient of pheromone. They also exhibit a broader projection than wild type cells, and reorient their polarity frequently, without regard for the gradient of pheromone. Evidence suggests that actin distribution may be affected. Actin polymerization is promoted by formins when bound by active Cdc42. Yeast have two formins, Bnr1 and Bni1 that are thought of as genetically redundant proteins, because the deletion of either protein does not lead to arrest of cell division. While they have functional overlap, other evidence suggests their functions are distinct. During mitosis, Bnr1 associates with the bud neck, while Bni1 associates with the polar cap in the emerging bud. We hypothesize that this hyperactive mutant Gα is able to direct more Bni1 to a larger area of the plasma membrane. If this were to happen, we predict the cell would then be able to polymerize actin cables to a broader area, resulting in a less focused polarized growth and the observed gradient tracking defects.

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1.0 Introduction

Cell survival requires interaction with the environment, responding to external stimuli with an appropriate intracellular response. This involves cell surface receptors, most commonly coupled to heterotrimeric G proteins. These G protein-coupled receptors (GPCRs) mediate responses to a variety of stimuli in humans, including light, odor, taste, hormones, and neurotransmitters (Neves, 2002). Simple eukaryotes use similar mechanisms to mediate cell motility, development, and sexual reproduction (Wang, 2008). The yeast *Saccharomyces cerevisiae* uses a GPCR to direct the pheromone response pathway (Dohlman and Thorner, 2001).

GPCR pathways are conserved in most eukaryotes, making yeast a good model organism for studying more complex biological systems, such as humans. The pheromone response pathway in *Saccharomyces cerevisiae* is one of the best characterized G protein signaling systems (Dohlman and Thorner, 2001). Haploid yeast detect and respond to pheromone gradients produced by the opposite mating type to find a mating partner (Segall, 1993). At a high dose of pheromone, yeast will form a short, focused mating projection in order to mate with yeast that are close by. At lower doses of pheromone, the yeast form a broader projection which grows towards the source of pheromone. The pheromone is detected by a GPCR, Ste2 (Bender A, Blumer KJ), which initiates the production of new gene products for mating, and polarizes the cytoskeleton in the direction of the source of the pheromone, facilitating gradient tracking.

The yeast pheromone receptor (Ste2) activates a large heterotrimeric G protein. Heterotrimeric G proteins are comprised of three subunits: The α-subunit that binds directly to GTP and hydrolyzes it to GDP, and a dimer consisting of the β- and ϒ-

subunits, which are sequestered by the inactive Gα (Dohlman and Thorner, 2001). When the pheromone receptor activates the Gα subunit (Gpa1), Gα binds GTP and dissociates from the GβϒDimer (Ste4/Ste18). The free GβϒDimer initiates two effector pathways: activation of a mitogen-activated protein kinase (MAPK) leading to transcriptional induction, and activation of the small G protein Cdc42, which promotes actin polymerization and cell polarization (Dohlman and Thorner, 2001). The GPCR pathway is desensitized by a Regulator of G protein Signaling (RGS) protein (Sst2), which acts as a GTPase-activating protein (GAP). The rate of GTP hydrolysis by Gα is enhanced by Sst2, thereby terminating the signal (Dohlman et al., 1996; Apanovitch et al., 1998).

When GβϒDimer is free to signal, it promotes activation of the guanine nucleotide exchange factor Cdc24 which activates Cdc42 (Butty, A.C, 1998; Nern, A., 1999). Accumulation of active Cdc42 causes morphological changes, leading to cell expansion towards the source of pheromone (gradient tracking) (Dohlman and Thorner, 2001). Collectively, Cdc42 and the accompanying polarization machinery are known as the polar cap (Slaughter, B.D., 2009). Critical for proper cell polarization is Gα.

Cells expressing a mutant Gα that cannot be turned off ($Gpa1^{G302S}$) are unable to track a gradient of pheromone. These cells exhibit a broader projection than wild type cells, and reorient their polarity frequently, without regard for the gradient of pheromone (DiBello, 1998). It is not clear why this mutant Gα causes gradient tracking defects. Cells containing the mutation exhibit abnormal septin structures, and an unusual broadening in the distribution of the exocytic marker (Kelley, 2015), providing evidence that actin localization may be effected. Among the proteins associated with the polar cap are Cdc42

effector proteins that carry out the work of polarizing the actin cytoskeleton, such as formins.

Formins promote actin polymerization when bound by active Cdc42 (Adams, 1990; Evangelista, 1997). Formins are actin organizing proteins, characterized by the presence of an FH2 domain, which is necessary for actin filament assembly (Evangelista et al., 1997, 2002; Pruyne et al., 2002; Sagot et al., 2002a,b). Yeast have two formins, Bnr1 and Bni1 that are thought of as genetically redundant proteins, because the deletion of either protein does not lead to arrest of cell division (Kohno, H.,1996; Imamura, 1997). While they have functional overlap, other evidence suggests their functions are distinct. During mitosis, Bnr1 associates with the bud neck, while Bni1 associates with the polar cap in the emerging bud (Buttery, 2007). During the pheromone response, the Gα recruits a pheromone specific kinase that phosphorylates Bni1, promoting association with the plasma membrane (Metodiev, 2002; Matheos, 2004; Fig.1). Bnr1 is not phosphorylated as part of the pheromone specific response.

Together these data suggest that, although the two formins have some redundant functionality, the cell uses them for different roles in mitosis and in the pheromone response. Since Bni1 is believed to be critical during the pheromone response, it's distribution should be affected in the mutant strain. We hypothesize that this hyperactive mutant Gα is able to recruit more kinase across a broader area of the plasma membrane, and thus direct more Bni1 to a larger area of the plasma membrane. If this were to happen, we predict the cell would then be able to polymerize actin cables to a broader area, resulting in a less focused polarized growth and the observed gradient tracking defects.

2.0 Literature Review

2.1 G protein coupled receptors

G protein coupled receptors (GPCRs) are seven-transmembrane domain receptors that sense extracellular signals and activate intracellular G-protein-mediated signal transduction pathways. They are the largest family of transmembrane receptors and are able to transmit external signals to intracellular responses by stimuli including light, protons, Ca2+, odorants, amino acids, nucleotides, proteins, peptides, steroids, and fatty acids (Maller, 2003).

2.2 Heterotrimeric G-proteins

Heterotrimeric G-proteins act as molecular switches, activated by ligand-bound receptors at the cell surface (Sprang, 1997). They are comprised of three subunits, the α, β and γ. The GPCR acts as a guanine nucleotide exchange factor (GEF) for Gα. Once pheromone is bound, the receptor catalyzes the exchange of GDP for GTP in the α-subunit of the heterotrimeric G-protein, resulting in the dissociation of the Gβγ from the Gα. (Dohlman and Thorner, 2001). The Gβγ and Gα are able to activate downstream effectors until Gα hydrolyzes GTP to GDP, sequestering the Gβγ dimer.

Regulators of G protein signaling (RGS) proteins act as GTPase-Activating Proteins (GAP), accelerating the GTPase activity of heterotrimeric G proteins. (Neves, 2002). RGS proteins also have the ability to interact directly with GPCRs (Hague et al., 2005; Kovoor et al., 2005; Ballon et al., 2006). This interaction presumably enhances the activity of the RGS protein with the Gα subunit (Ballon et al., 2006).

When Gα is inactivated (GDP-bound) it rapidly reassociates with Gβγ, ending the pheromone response. Thus Sst2 is required for signal termination and pathway desensitization.

2.3 Pheromone response pathway

G protein signaling systems are conserved among eukaryotes. One of the best characterized is the pheromone response pathway in *Saccharomyces cerevisiae*. This pathway begins when a GPCR, Ste2, binds to peptide pheromones. Pheromone receptors are delivered to the cell surface via the secretory pathway but are later internalized and delivered to the vacuole for degradation. Both processes are accelerated by pheromone stimulation (Jenness and Spatrick, 1986).This receptor activates a large G protein composed of an Gα subunit, Gpa1, and a Gβγ dimer, Ste4 and Ste8 (Wang, Y., and Dohlman, H.G. (2004). Pheromone signaling mechanisms in yeast: a prototypical sex machine. Science 306, 1508–1509.) Once activated, Gpa1 becomes GTP bound and dissociates from the Gβγ dimer (Dohlman and Thorner, 2001).

The free Gβγ dimer initiates two effector pathways: the first is a MAP kinase cascade leading to the transcription of gene necessary for cell fusion and growth arrest, and the second triggers Cdc42-dependent morphological changes (Dohlman and Thorner, 2001). The second pathway leads to proper expansion towards a gradient of pheromone (Arkowitz, R.A., 2009; Park, H.O., and Bi, E., 2007) once the guanine nucleotide exchange factor Cdc24 is recruited by Gβγ, so Cdc42 activation is spatially coupled to sites of receptor activation (Butty, A.C., 1998; Nern, A., 1999). Activated Cdc42 defines the polarity of the cell by promoting actin polymerization and exocytosis (Bi, E., 2012).

Collectively, Cdc42 and accompanying machinery are known as the polar cap (Slaughter, B.D., 2009).

This pathway shares components with the mitosis, budding, machinery. While bud site formation occurs in response to an internal static queue (Casamayor, A., 2002), chemotropic growth is dynamic, adapting to changing external signals (Arkowitz, R.A., 2009; Moore, T.I., 2013).

Cells exposed to high doses of pheromone arrest in the G1 phase of the cell cycle and polarize their growth, forming a mating projection or "shmoo,"acting as the site of contact and fusion between mating partners. At lower doses of pheromone, haploid cells transiently arrest in the G1 phase of the cell cycle and exhibit hyperpolarized or elongated growth. Upon reentering the cell cycle, they exhibit a polar rather than an axial budding pattern (Erdman and Synder, 2001). Growth in the direction of increasing pheromone concentration is considered "chemotropic" (Segall, 1993; Paliwal et al., 2007; Hao et al., 2008).

2.4 Polar cap mobility

The RGS protein, Sst2, promotes polarized cell expansion by organizing the localization of cytoskeletal scaffolding proteins called septins (Gladfelter, A.S., 2001; Caudron, F., 2009). During mitosis, septins form a double-ring structure at the mother-daughter bud neck, acting as a diffusional barrier between the two cells (Takizawa, P.A., 2000) while constraining the movement of the polar cap (Barral, Y., Mermall, V., 2000; Okada, S., 2013). In shmooing cells, septin bundles form the base of the mating projection (Giot, L., 1997; Longtine, M.S., 1998).

GAP activity of Sst2 is required to maintain separation of the polar cap and septins. If GAP activity is absent, septins distribute asymmetrically and the polar cap follows. Polar cap movement is limited by Sst2, preventing aberrant turning from the pheromone gradient (Kelley et al., 2015).

2.5 Gpa1 G302S mutation

When the G302S mutation is introduced into Gpa1, cells are unable to track a gradient of pheromone, exhibit a broader projection than wild-type, and reorient their polarity frequently (DiBello, 1998). Cells containing the unGAPable $Gpa1^{G302S}$ mutation show abnormal septin structures and aberrant colocalization of the polar cap and septins (Kelley et al., 2015). These cells also have an unusual broadening in the distribution of the exocytic marker (Exo84), but not when compared to itself, indicating that the observed effects are due to increased variability in the site of exocytosis relative to the polar cap (Kelley et al., 2015).

2.6 Formins

Formins are a family of proteins characterized by an FH2 domain, which is needed for actin filament assembly (Evangelista et al., 1997, 2002; Pruyne et al., 2002; Sagot et al., 2002a,b; reviewed in Faix and Grosse, 2006; Kovar, 2006; Fig.2). Actin filaments are nucleated by the FH2 domain, and in the presence of the capping protein, enables barbed end elongation (Zigmond et al., 2003; Harris et al., 2004; Moseley et al., 2004; Kovar, 2006).Formins can attach processively to the barbed end (Pruyne et al., 2002; Higashida et al., 2004; Kovar and Pollard, 2004; Romero et al., 2004), allowing them to generate linear arrays of actin filaments, including actin cables or the cytokinetic

ring (Kovar, 2006). Formins also contain an FH1 domain that binds profilin (Chang et al., 1997; Evangelista et al., 1997; Watanabe et al., 1997; Fig.2), which facilitates actin subunit delivery to the barbed ends of actin filaments capped with formins (Sagot et al., 2002b; Kovar, 2006).

In *Saccharomyces cerevisiae* there are two formins, Bni1 and Bnr1, that are responsible for nucleating and elongating actin filaments. These are organized into two actin structures: actin cables that are polarized along the mother–bud axis and act as tracks for myosin mediated delivery of vesicles and organelles to the bud, and the actomyosin ring at the mother–bud neck that contributes to cytokinesis (Vallen et al., 2000). They are considered to be genetically redundant, for deletion of either is not significantly deleterious to cell growth (Kohno et al., 1996; Imamura et al., 1997), while loss of both genes is lethal (Ozaki-Kuroda et al., 2001). Some evidence suggests they have distinct functions. Bnr1 localization is septin dependent (Pruyne et al., 2004; Buttery et al., 2007; Gao et al., 2010) and occurs exclusively to the bud neck (Buttery, 2007). Fus3 binding to Gpa1 and phosphorylation of Bni1 facilitates pheromone-induced morphogenesis (Metodiev et al., 2002; Matheos et al., 2001; Fig.1), and during mitosis Bni1 turns over rapidly at the bud tip and bud neck (Buttery, 2007).

Currently it is unclear if Gpa1, Fus3, and Bni1 form a stable complex or transiently associate in vivo. One suggested possibility is that Gpa1, Fus3, and Bni1, through interactions with Rho-1, are required for generating actin cables, while Gβγ-Cdc24-Far1 with Cdc42 are responsible for restricting the location of actin cables on the cell cortex (Arkowitz, 2009).

Other research has suggested that there is a competition between the heterotrimeric G-protein and sensing-independent Cdc42 activity for influence over the spatial dynamics of the polar cap. When there is a 180^{o} switch in the gradient of pheromone, wild-type cells display either projection bending growth or second-projection formation, depending on the pheromone concentration. When heterotrimeric G-protein signaling is hyperactive, projection bending is promoted, while hyperactive Cdc42 signaling promotes second projections. If Bni1 is overexpressed, the mutant exhibits characteristics of both persistent polarization (projection bending) and dynamic repolarization (second projection formation) at low and high α-factor. This provides evidence that Bni1 is a limiting factor, and at wild-type levels can restrict cells to a single polar cap and a single behavior at a given concentration of pheromone (Moore, 2012).

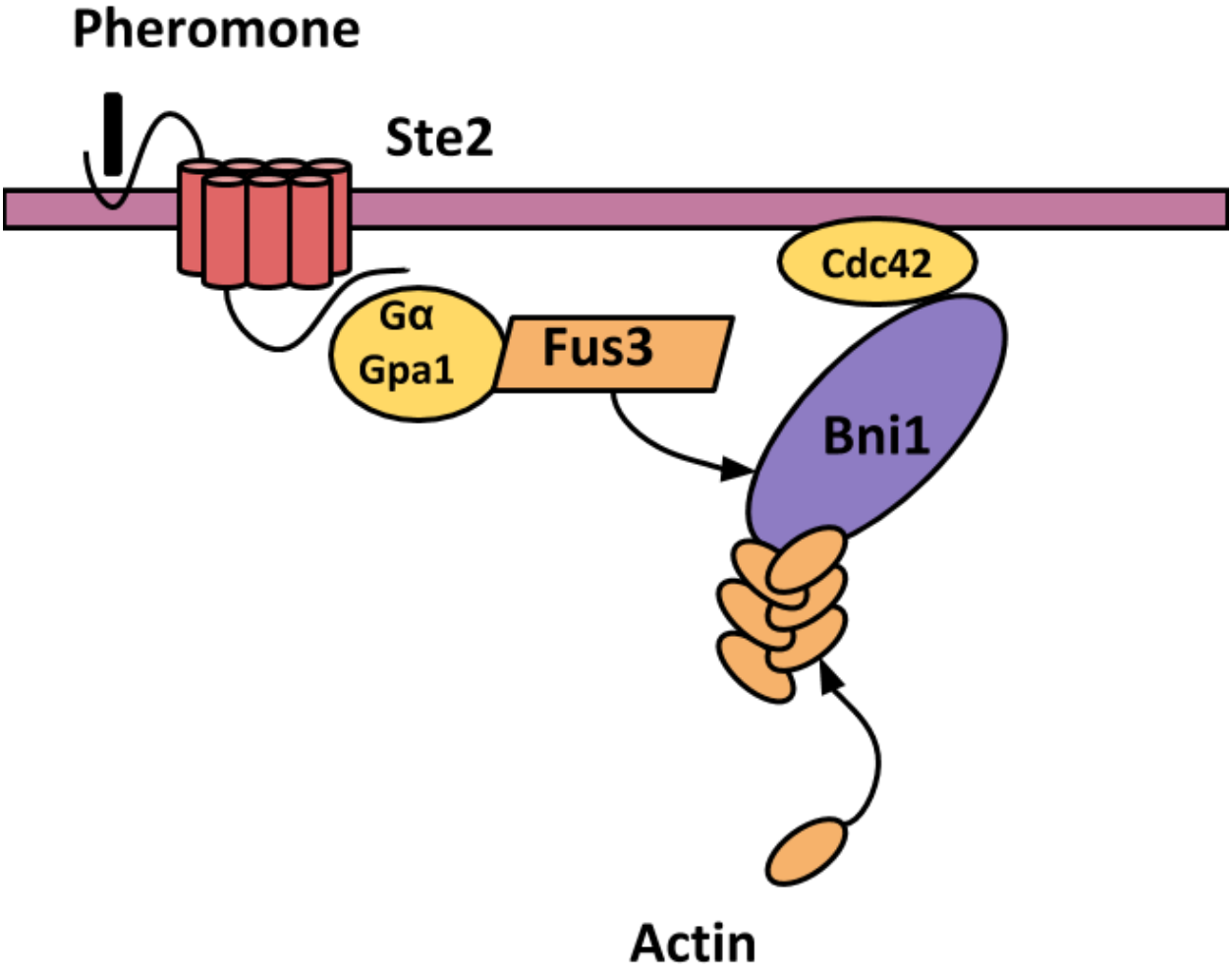

Figure 1. Part of the yeast pheromone response pathway leading to morphogenesis. It begins when pheromone binds to the receptor (Ste2), which activates Gα (Gpa1). Gα recruits a pheromone specific kinase (Fus3) which phosphorylates Bni1, promoting its association with the plasma membrane through recruitment by a small G-protein (Cdc42). Once associated, Bni1 is able to polymerize actin

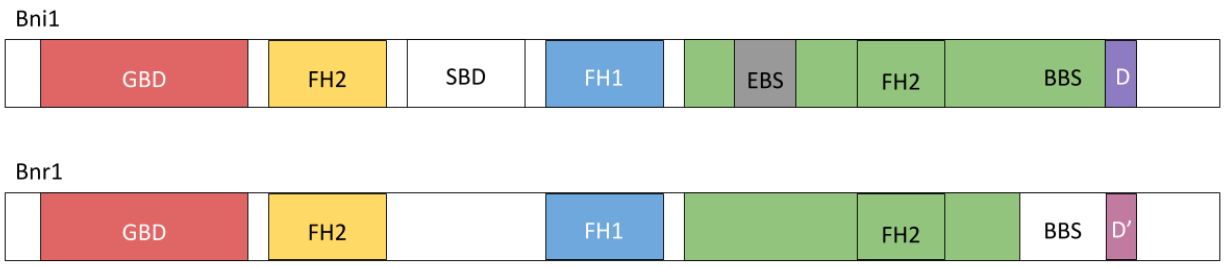

Figure 2. Schematic of yeast formins Bni1 and Bnr1, indicating domain locations. Both contain a FH2 and FH1 domain, characteristic of formins. GBD = GTPase binding domain, Spa2-binding domain, FH1 = Proline-rich formin homology 1 domain, EBS = eEF1A-binding site, FH2 = Formin-homology 2 domain, BBS = Bud6p-binding site, D = Dia-autoregulatory domain (DAD), D' = DAD-like sequence.

3.0 Materials and Methods

3.1 Strain Construction

All cells were grown at 30°C in yeast extract peptone medium (YPD) or synthetic complete medium containing 2% (w/v) dextrose. Plasmid-transformed cells were grown in synthetic complete medium that lacked the appropriate nutrient. Standard procedures for the growth, maintenance, and transformation of yeast and bacteria were used throughout.The yeast (*Saccharomyces cerevisiae*) strains were all constructed using the BY4741 background. Strains and oligonucleotides used are listed in Table1 and Table2 respectively. The *Bni1-GFP::His3* strain was from the GFP collection. *Bni1Δ* and *Bnr1Δ* strains were from the yeast knockout collection.

The *Bnr1-GFP::KanMX6* strain was made by PCR amplification. Gene-specific oligonucleotide primers were synthesized, each of which had been designed to share complementary sequences to the GFP tag-marker cassette at the 3' end and contain 40 base pairs (bp) of homology with a specific gene of interest to allow in-frame fusion of

the GFP tag at the C-terminal coding region of the gene. Gene-specific cassettes containing a C-terminally positioned GFP tag were then generated by PCR using as a template

pFA6a–GFP(S65T)–KanMX6, which permits selection of transformed strains in media containing geneticin. The haploid parent yeast strain BY4741 was transformed with the PCR products, and strains were selected in YPD medium (yeast extract peptone medium) containing geneticin (G418). Insertion of the cassette by homologous recombination was verified by genomic PCR of samples from individual colonies with a primer internal to the GFP tag and a separate set of ORF-specific primers designed to produce a product of approximately 500 bp.

Table 1. Strains Used

Strain	Parent	Description
BY4741		*Mata leu2Δ met15Δ his3Δ ura3Δ*
Bni1-GFP	BY4741	*Bni1-GFP::HIS3*
Bnr1-GFP	BY4741	*Bni1-GFP::KanMX4*
Bni1Δ	BY4741	*Bni1Δ::HIS3*
Bnr1Δ	BY4741	*Bnr1Δ::HIS3*

Table 2. Oligonucleotides Used

Oligonucleotide Name	Sequence	Gene	Description
SSM-1	AGAGAGAACGCATGCTATGCTGAACGATATTCAAAATATAGGTGACGGTGCTGGTTTA	Bnr1	pFA6-GFP-KanMX forv for tagging Bnr1 with G
SSM-2	TTATATAAGCTCCACAACTACATAAAATACTAAGTCTTCATCGATGAATTCGAGCTCG	Bnr1	pFA6-GFP-KanMX rev for tagging Bnr1 with G
SSM-3	GTGCCAAGGAAAACATTGA	Bnr1	Verification of Bnr1 3′ t primer
SSM-4	ACAGACACATTGCCCATCTT	Bnr1	Verification of Bnr1 3′ t primer

3.2 Microscopy

Live cell microscopy was performed using an Olympus IX83 inverted microscope using a 60x 1.49 NA objective, Excelitas X-Cite 120LED Boost lighting, a Photometrics Prime 95B sCMOS camera, and controlled by a Prior ProScan H31. The temperature of the specimen was maintained at 30°C using a Bioptechs Objective Heater.

Cells were imaged at 5 min intervals for 3 h on an agar pad made of SCD medium and α-factor pheromone as indicated. The 488 nm and 561 nm channels were collected as 8 Z-positions spaced 0.5 μm apart.

Single time point images of the Bni1-GFP and Bnr1-GFP strains were taken on agar pads (2% agar in SCD) with 3 μM pheromone. A much higher concentration of pheromone was used on the agar pads because the accumulation of the Bar1 protease results in a lower effective concentration of pheromone on the agar pad.

3.3 Image Analysis

Image analysis was performed using FIJI (FIJI Is Just ImageJ, http://fiji.sc/Fiji [Schindelin, J.,]). Images were processed in the following manner using FIJI: LUT was inverted, Background Subtraction was used with a rolling ball radius of 20 pixels and a light background, Brightness/Contrast was adjusted, and a maximum intensity projection was performed on z-stacks.

4.0 Results

4.1 Formin Deletion Mutants Respond Differently to Pheromone

During the yeast pheromone response pathway, Fus3 binds directly to the activated Gpa1 (Metodiev et al., 2002; Fig.1). This positions Fus3 to phosphorylate the formin Bni1, which can promote actin assembly and polarized growth (Matheos et al., 2004; Fig.1). To see if Bnr1 can fulfill this role in the absence of Bni1, deletion mutants for both formins, Bni1 and Bnr1, were looked at during mitosis and in the presence of pheromone. Both formin deletion mutants are able to proceed through the cell cycle and bud (Kohno, H.,1996; Imamura, 1997; Fig. 3). In the presence of pheromone, Bnr1Δ is able to shmoo normally, as compared to wild-type (Fig. 4). Bni1Δ is unable to shmoo properly, forming a mating projection that is broad and less focused as compared to wild-type (Fig. 4).

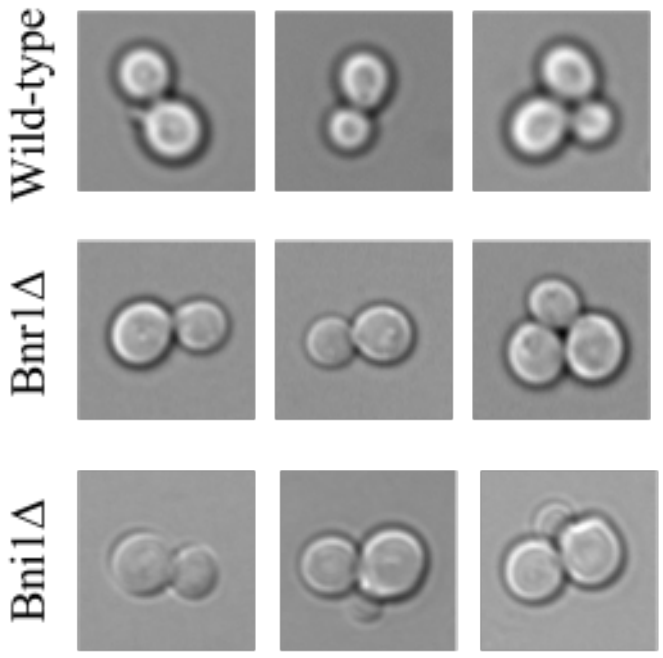

Figure 3. DIC images of wild-type (top), Bnr1Δ (middle), and Bni1Δ (bottom) in

budding cells. Compared to wild-type, Bnr1Δ and Bni1Δ mutants both show normal morphology and are able to proceed through mitosis.

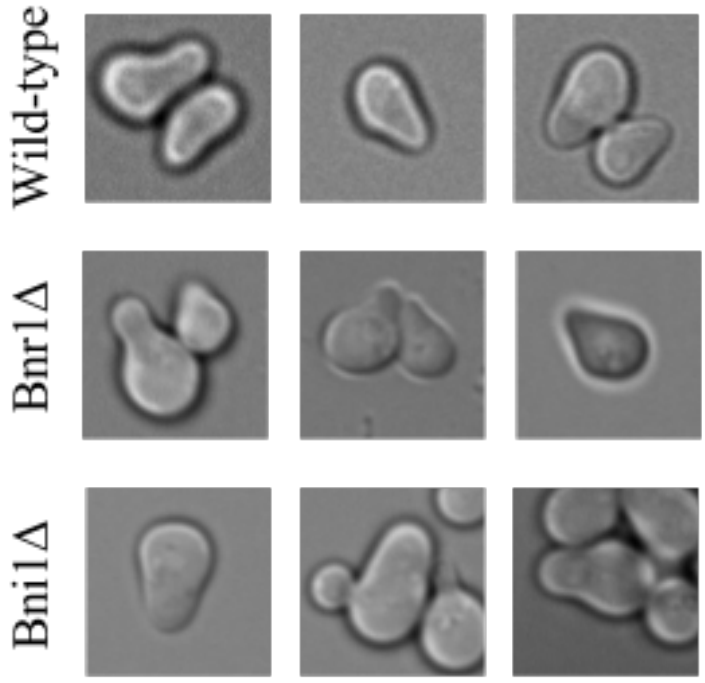

Figure 4. DIC images of wild-type (top), Bnr1Δ (middle), and Bni1Δ (bottom) in the presence of 3 μM α-factor pheromone. Compared to wild-type, Bnr1Δ mutants shows normal morphology and is able to shmoo. Bni1Δ mutants show a broadened projection as compared to wild-type.

4.2 Bni1-GFP and Bnr1-GFP Localize Differently During Mitosis and the Pheromone Response

In mitotic cells, Bnr1 localizes to the bud neck (Kamei et al., 1998; Pruyne et al., 2004a, Fig. 5) and remains static there (Buttery et al., 2007) as seen by Bnr1-GFP localization. Bni1-GFP has previously been shown to display rapid turnover at the bud tip and bud neck during different phases of mitosis cycle (Ozaki-Kuroda et al., 2001; Pruyne

et al., 2004a; Buttery et al., 2007). Bni1-GFP can be seen at both the bud neck and bud tip (Fig. 5). In the presence of pheromone no localization is observed for Bnr1-GFP, while Bni1-GFP localizes to the polar cap at the shmoo tip (Fig. 6).

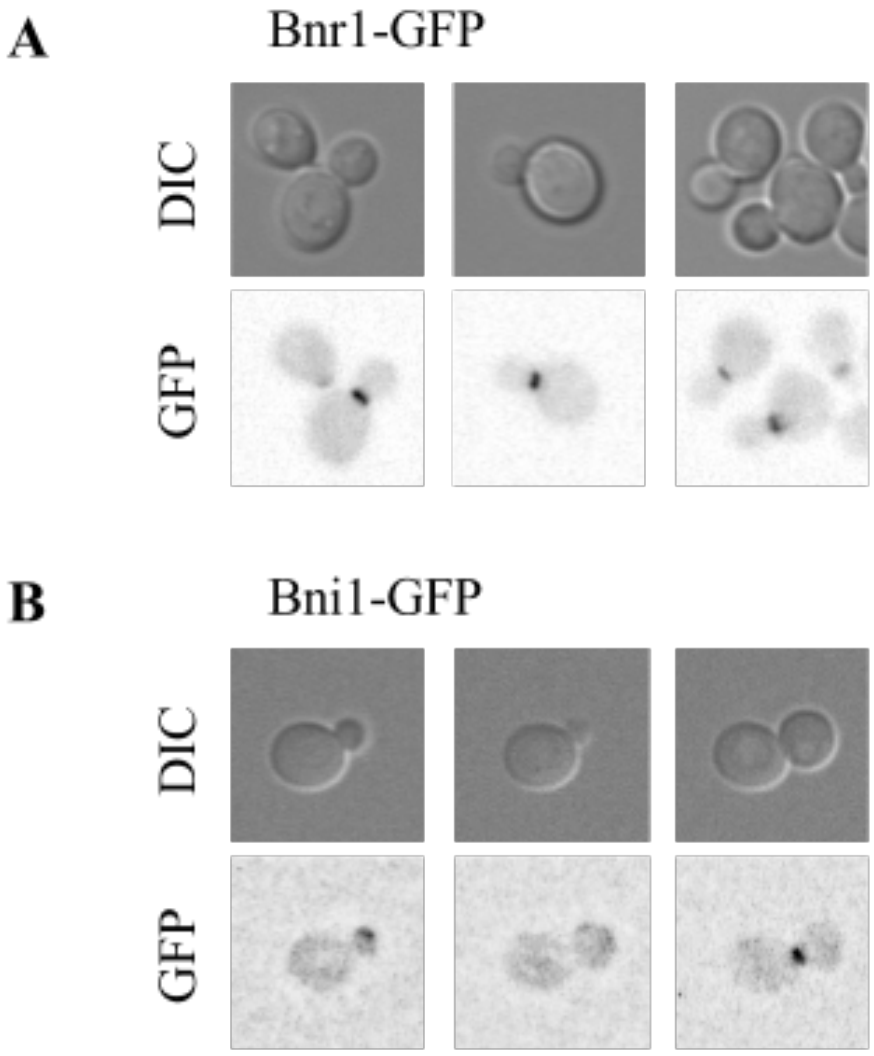

Figure 5. Localization of Bnr1-GFP and Bni1-GFP in budding cells. Bnr1-GFP localizes to the bud neck and remains static there (A). Bni1-GFP displays localization to both the bud tip and bud neck at different times during mitosis (B).

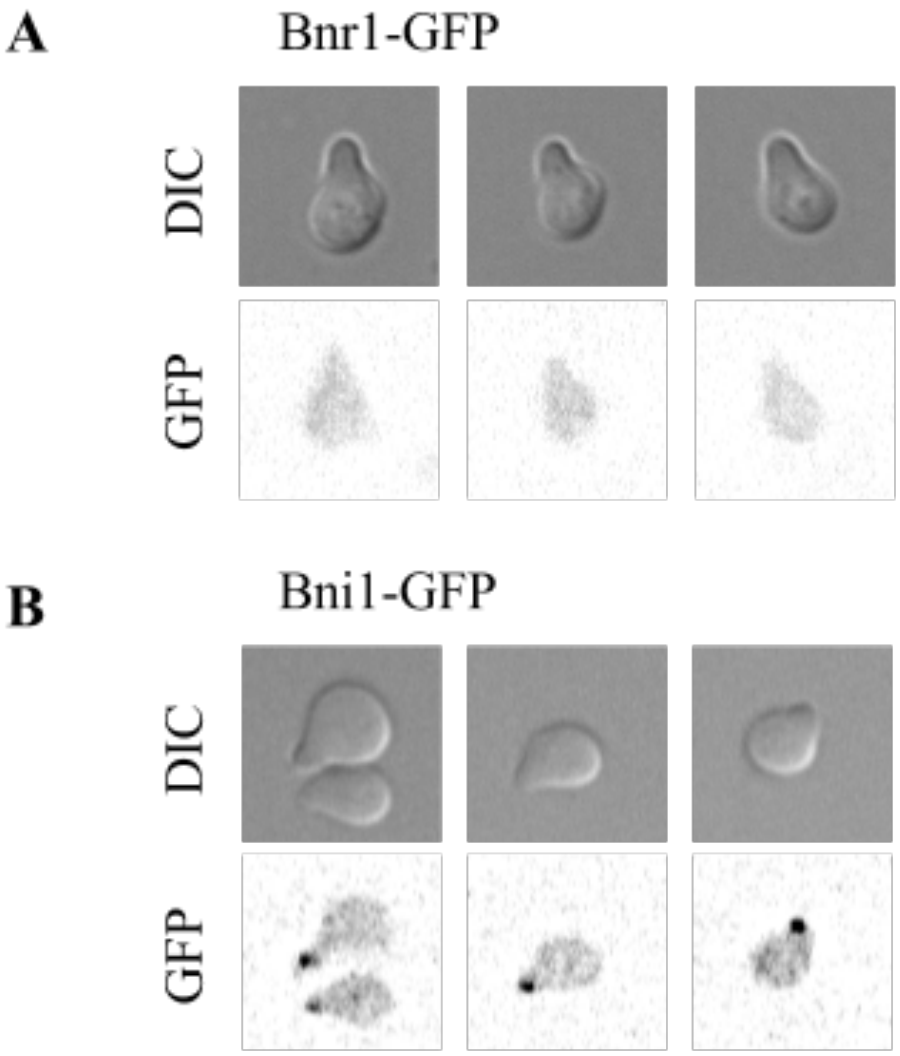

Figure 6. Localization of Bnr1-GFP and Bni1-GFP in shmooing cells. Bnr1-GFP shows no localization in the presence of pheromone (A). Bni1-GFP displays tight localization to the shmoo tip, at the polar cap (B).

5.0 Discussion

The yeast pheromone response pathway in *Saccharomyces cerevisiae* is a well characterized G-protein signaling system (Dohlman and Thorner, 2001). GPCRs are conserved in most eukaryotes, making *S. cerevisiae* a good model organism to explore more complex systems, such as humans. To better understand how these pathways function, it is important to determine how haploid yeast are able to detect and respond to pheromone to further characterize this signaling system so it can be applied to related ones. This goal of this study was to characterize the yeast formins, Bni1 and Bnr1, and determine if they are genetically redundant. While this was previously thought, as deletion of either does not lead to growth cycle arrest (Kohno, H.,1996; Imamura, 1997), other evidences suggest they may have distinct roles within the cell that may be defined by the presence or absence of pheromone.

5.1 Bni1 is essential for shmoo formation

As compared to wild-type, Bnr1Δ mutants are able to form the mating projection normally (Fig. 4). This indicates that Bnr1 is not necessary for shmoo formation. Bni1Δ mutants are unable to form a proper mating projection, having a broadened projection, as compared to wild-type (Fig. 4). This supports the claim that Bni1 is necessary for the pheromone response, as the mating projection is not as focused in Bni1Δ mutants.

Localization of Bnr1-GFP and Bni1-GFP was also compared in mitotic and shmooing cells. Bnr1-GFP is static at the bud neck during mitosis (Buttery et al., 2007; Fig. 5), but does not localize in shmooing cells (Fig. 5). Bni1-GFP displays rapid turnover at both the bud neck and bud tip (Buttery et al., 2007; Fig. 5), and localizes to the shmoo tip during the pheromone response, remaining static there (Fig. 5).

This difference in localization between the two formins suggest they possess distinct functions, and are not entirely redundant proteins. Although there may be some functional overlap, it is evident that Bnr1 is more involved in yeast budding due to its static localization to the bud neck throughout mitosis (Fig. 6). Furthermore, no localization of Bnr1-GFP is observed in shmooing cells (Fig. 6), indicating it is likely not involved in the recruitment of actin to the polar cap during the pheromone response. While Bni1 is involved in mitosis, as Bni1-GFP displays rapid turnover between the bud neck and bud tip (Ozaki-Kuroda et al., 2001; Pruyne et al., 2004a; Buttery et al., 2007), it does not display the static behavior of Bnr1-GFP. Bni1 is also known to be tightly regulated by the pheromone response pathway, as phosphorylation by the pheromone-specific kinase, Fus3, promotes association of Bni1 with Cdc42 at the plasma membrane where it is able to recruit actin and promote polarized growth (Matheos et al., 2004). In the presence of pheromone Bni1-GFP localizes to the shmoo tip, tightly localized to the polar cap (Fig. 6), suggesting that Bni1 is required for proper shmoo formation.

5.2 Conclusions and Future Work

The G-protein signaling system that is the yeast pheromone response pathway is well characterized in *Saccharomyces cerevisiae*. What is not well understood is why yeast containing a $gpa1^{G302S}$ mutation are unable to track a gradient. It was previously observed that exocytosis is altered in the mutant strain (Kelley, 2015), leading us to conclude that actin distribution may also be. Actin polymerization is controlled by formins, in *S. cerevisiae*, Bni1 and Bnr1. Although believed to be genetically redundant, we believed that they have distinct functions. Bnr1 was shown to localize to the bud neck during mitosis, but not to localize during shmooing. Bni1 does not localize to one

location during mitosis, but localizes tightly to the polar cap during shmooing. Bni1Δ and Bnr1Δ strains both are able to undergo mitosis, but shmoo formation is disrupted in Bni1Δ only. This supports the claim that Bni1 and Bnr1 have distinct functions, and the Bni1 is more involved in the pheromone response.

To further support the claim that Bni1, and not Bnr1, is essential for the pheromone response pathway, gradient tracking experiments could be performed for both strains. Using a microfluidics device, a gradient of pheromone could be created across a chamber containing yeast cells from low to high pheromone. Over the course of 12 hrs, at 5 min intervals, images would be taken to observe how the deletions mutants respond to a gradient of pheromone. If Bnr1Δ mutants were able to track the gradient properly, but Bni1Δ mutants were unable to, this would support the claim that Bni1 is essential for responding to pheromone while Bnr1 is not. Actin localization in both deletion mutants would also be useful in determining how actin distribution is altered in the Bni1Δ mutant as compared to the Bnr1Δ mutant. This could be done by transforming deletion mutants with Lifeact-GFP plasmid that allows for live staining of actin.

Localization of Bni1-GFP and Bnr1-GFP could be looked at in the presence of pheromone over a time course to observe their localizations over time. A longer time period would allows us to see Bnr1-GFP move from the bud neck in mitotic cells to no localization in shmooing cells, and see if Brn1-GFP localizes at all while responding to pheromone.

Future experiments could be done to confirm that Bni1 localization is altered in $gpa1^{G302S}$ mutants. To confirm if Bni1 localization is altered, Bni1-GFP profiles in wild-type and in the mutant strain need to be generated and normalized spatially to a polar cap

marker, like Bem1, after exposure to uniform pheromone. From this, the median distance of Bni1 from the center of the polar cap in both strains can be determined to observe if the distribution is broadened.

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