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Candida albicans Adenylyl cyclase – cAMP Pathway and Hyphal Growth

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Abstract of the Dissertation

***Candida albicans* Adenylyl cyclase – cAMP Pathway and Hyphal Growth**

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The human opportunistic fungal pathogen *Candida albicans* can undergo a transition from budding to hyphal growth in response to host cues that promotes tissue invasion and dissemination through the host. Accompanying the morphogenic change is an upregulation of genes that promote virulence. Morphogenic switching and subsequent gene regulation are required for full virulence in the host and are an area of intense study. It has been shown that increased production of cAMP by Cyr1 Adenylyl cyclase can induce hyphal growth. Further, it was proposed that activation of Cyr1 and an increase in cAMP were necessary for all hyphal growth in *C. albicans*. The main evidence for this necessity is that cells lacking Adenylyl cyclase (*cyr1Δ*) fail to induce hyphal growth in response to most physiological inducers. This evidence is not conclusive as deletion of *CYR1* severely affects cellular health. I have demonstrated that signaling through Cyr1 and cAMP are not required for hyphal morphogenesis or virulence gene induction. I propose that cAMP-independent pathways are responsible for much of the morphogenic and gene regulatory signals produced by physiological inducers. My findings shed light on hyphal signaling in *Candida albicans* and will aid in the identification of drug targets and the development of novel therapeutics.

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List of Abbreviations:

cAMP: cyclic-AMP (Adenosine Monophosphate)

db-cAMP: dibutyryl cyclic-AMP

Dex: Dextrose

GlcNAc: N-acetylglucosamine

Gal: Galactose

HSGs: hyphal-specific genes

PR: Pseudorevertant

Ura: Uracil

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Chapter 1. Introduction

1.1 *Candida albicans* – The most common human fungal pathogen

Candida albicans is the causative agent of most medically relevant mycoses (Wisplinghoff *et al.*, 2004). Though it is a normal member of the gastrointestinal flora, inability of the host immune system to control its growth or other conditions that promote overgrowth of *C. albicans* can lead to painful mucosal infections or deadly systemic infections (Odds, 1988, Heitman *et al.*, 2006).

Systemic infections begin when *C. albicans* reaches the bloodstream; a condition known as candidemia (Sudbery, 2011). Budding cells are roughly the size of red blood cells, allowing their dissemination through the body to distant sites through the vasculature. Escape from the bloodstream and invasion of tissues, even solid organs, is accomplished when budding cells switch to hyphal growth and induce hyphal-specific genes (HSGs). (Whiteway & Oberholzer, 2004, Kumamoto & Vinces, 2005, Blankenship & Mitchell, 2006). When fungal cells penetrate organs, the disease then becomes known as disseminated candidiasis, which has a 40% attributable mortality even with current antifungal therapies (Phan *et al.*, 2000, Wisplinghoff et al., 2004).

In vitro studies have demonstrated that invasive growth capable of penetrating epithelial and endothelial layers is a characteristic solely of the hyphal morphology (Dalle *et al.*, 2010). This agrees well with what is seen in a true infection: though cells at the infected site are found in budding, pseudohyphal, and hyphal forms, only hyphal forms are found penetrating epithelial cells (Scherwitz, 1982).

1.2 *Candida albicans* Hyphal growth

Candida albicans hyphal growth is a highly polarized growth form. Various stimuli mimicking the host environment including serum, alkaline pH, CO_2, physiological temperature, bacterial peptidoglycan breakdown products (MDPs), and N-acetylglucosamine (GlcNAc) induce *C. albicans* to switch to filamentous pseudohyphal and hyphal growth *in-vitro*, and upregulate virulence factors (Biswas et al., 2007; Whiteway and Bachewich, 2007; Davis, 2009; Sudbery, 2011; Wang, 2013). When budding cells are exposed to these hyphal inducers they will not form the usual bud, but instead form a cylinder-like protrusion, called a germ tube, that grows by elongation at the tip (**Figure 1-1**).

The molecular mechanism of hyphal growth, which is a form of highly polarized growth, is well understood. Polarized growth requires secretory vesicle delivery to be restricted to the site of polarized growth. Studies have shown that treatment with Latrunculin A or Cytochalasin A, which cause a dispersal of filamentous actin, interfere with this delivery, and cause vesicles to be delivered in a wider area, resulting in isotropic growth much like that of budding cells (Crampin *et al.*, 2005, Wisplinghoff et al., 2004, Hazan & Liu, 2002). This indicates that actin polarization is required for polarized growth, and it is thought that secretory vesicles travel along actin cables to sites of polarized growth (Sudbery, 2011).

Critical to the restriction of vesicle delivery to the hyphal tip is the phosphorylation of GEF Sec2 by the Hgc1-Cdc28 cyclin-dependent kinase complex. Sec2 is the GEF of Sec4, a Rab-type GTPase, and hyphal growth requires that it remain localized to a fine point on the hyphal tip, thereby directing vesicle delivery. Hgc1 is a G1 cyclin-like protein and, with the Cdc28 CDK cyclin-dependent kinase, maintains phosphorylation of Sec2 (Bishop *et al.*, 2010).

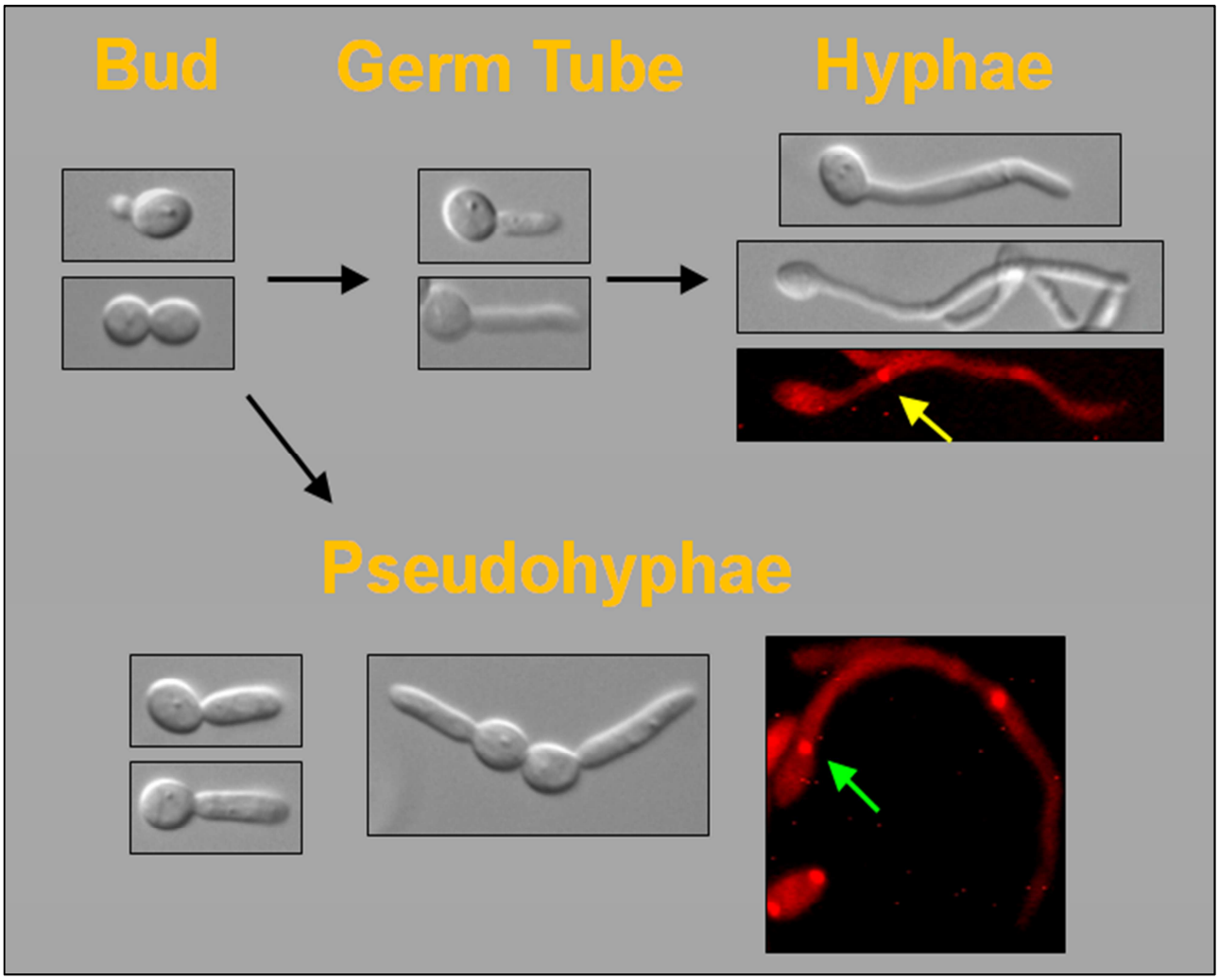

Figure 1-1: Examples of *Candida albicans* budding cells, germ tubes, hyphal, and pseudohyphal cells. Red stained cells were treated with Pontamine Fast Scarlet (see Chapter 3), a dye that stains the cell wall. The yellow arrow on the hyphal cell denotes the first septum, which presents as a bright staining area. Note it is distal to the budneck; a characteristic of true hyphae. The green arrow on the pseudohyphal cell denotes the first septum present at the budneck; a characteristic of pseudohyphal cells.

1.3 Hyphal-specific genes

Concomitant with the bud to hyphal switch is the induction of a set of characteristic genes. Determination of genes upregulated during induction of hyphal growth has been performed many times using different inducers and conditions (Kadosh & Johnson, 2005, Gunasekera et al., 2010, Nantel *et al.*, 2002). As mentioned before, various stimuli can cause

hyphal growth *in vitro* but several genes and classes of genes are induced whether Serum, GlcNAc, or other means are used; these are referred to as hyphal-specific genes (HSG's) (Berman & Sudbery, 2002). **Table 1-1** lists all genes modulated 3-fold or more during a GlcNAc induction of hyphal growth (Gunasekera et al., 2010). HSG's include but are not limited to Adhesins of the *ALS* family, the cell-wall anchored protein Hwp1, Secreted Aspartyl Proteases of the *SAP* family, antioxidant enzymes of the *SOD* family, and the previously mentioned G1-cyclin like protein *HGC1*. Adhesins and Hwp1 allow attachment to host cells during infection (Mayer *et al.*, 2013). Hwp1 is interesting in that it exploits host transglutaminase (TGase) to allow covalent linkage to host cells (Williams *et al.*, 2013). Sap5 is a member of the Secreted aspartyl proteases of which there are 10 encoded in the genome and are thought to contribute to virulence by degrading host structures (Naglik *et al.*, 2003). The SOD family of super oxide dismutases are thought to aid *Candida albicans* in combating oxidants produced by the immune system (Martchenko *et al.*, 2004, Gleason *et al.*, 2014). *HGC1* was discussed previously and is interesting in that it is the only HSG actually required for hyphal growth (Berman & Sudbery, 2002, Zheng & Wang, 2004).

ORF	Gene	Description	Fold
Upregulated 3-fold or more			
orf19.5392	***NGT1***	GlcNAc-specific transporter	**23.2**
orf19.2157	***DAC1***	GlcNAc-6-phosphate deacetylase	**22.3**
orf19.2156	***NAG1***	Glucosamine-6-phosphate deaminase	**21.7**
orf19.2154	***HXK1***	GlcNAc kinase	**7.5**
orf19.1066	***GIG1***	Induced by GlcNAc; localized in cytoplasm; mutation causes increased resistance to nikkomycin Z	**7.0**
orf19.2457	***Unnamed***	Predicted ORF	**5.5**
orf19.2833	***PGA34***	Putative GPI-anchored protein of unknown function	**5.1**
orf19.5585	***SAP5***	Secreted aspartyl proteinase: expressed in mucosal and systemic infection	**4.9**
orf19.3374	***ECE1***	Protein comprising eight 34-residue repeats, hyphal-specific expression	**4.2**
orf19.1816	***ALS3***	Adhesin; ALS family; role in epithelial adhesion, endothelial invasiveness; GPI-anchor cell wall protein	**4.2**
orf19.1822	***UME6***	Zn(II)2Cys6 transcription factor; role in hyphal extension, virulence,	**3.5**
orf19.1321	***HWP1***	Hyphal cell wall protein; can covalently crosslink to epithelial cells by host transglutaminase	**3.4**
orf19.6028	***HGC1***	Hypha-specific G1 cyclin-related protein involved in regulation of hyphal morphogenesis	**3.2**
orf19.1124	***Unnamed***	Predicted ORF	**3.2**
orf19.6202	***RBT4***	Similar to plant pathogenesis-related proteins; required for virulence in mouse systemic model	**3.1**
orf19.701	***CFL11***	Similar to ferric reductase Fre10p; flucytosine repressed; possibly adherence-induced	**3.0**
Downregulated 3-fold or more			
orf19.1709	***Unnamed***	Sterol carrier domain protein	**18.7**
orf19.3618	***YWP1***	Protein with suggested role in dispersal in host; mutation causes increased adhesion and biofilm formation	**9.1**
orf19.3548.1	***WH11***	Cytoplasmic protein expressed specifically in white phase yeast-form cells; expression in opaque cells increases virulence	**4.1**
orf19.3571	***Unnamed***	Predicted ORF	**4.0**
orf19.4629	***Unnamed***	Orthologues have ubiquitin ligase activity, role in mitochondrion inheritance, protein ubiquitination	**3.6**

Table 1-1: Transcriptional changes associated with GlcNAc induction of hyphal growth.
Data from (Gunasekera *et al*., 2010). Hyphal growth was induced by adding GlcNAc into cells growing at low density in buffered Galactose containing medium. Avoiding changes in pH and cell density while inducing hyphal growth with GlcNAc allows GlcNAc and hyphal growth specific changes to be detected.

1.4 Increased signaling through the cAMP pathway can cause hyphal growth in *Candida albicans*

The model that budding cells disseminate through vasculature and hyphal cells invade into deep tissues predicts that cells stuck in a single morphology, either budding or hyphal, would be attenuated for virulence. Indeed, it has been demonstrated that *C. albicans* cells locked in either budding or filamentous growth forms are attenuated for virulence (Bahn *et al.*, 2003, Zheng & Wang, 2004, Murad *et al.*, 2001). Thus, the molecular pathways by which *Candida albicans* senses the host environment and govern the bud/hyphal decision are critical to virulence and are an area of intense study.

Several central eukaryotic cell-signaling pathways conserved from single celled eukaryotes to humans regulate bud to hyphal morphological switching in *C. albicans*. The MAP Kinase Pathway regulates a downstream transcription factor, Cph1, which regulates both the bud to hyphal switch and expression of virulence factors (Monge *et al.*, 2006). Downregulation of signaling through the TOR pathway has been reported to be important for prolonged hyphal growth (Lu *et al.*, 2011). However, it is the Adenylyl cyclase – cyclic-AMP (cAMP) pathway that is most important to the hyphal switch and expression of HSGs (Rocha *et al.*, 2001).

Figure 1-2 shows a simplified view of the C. albicans cAMP pathway. The pathway has 10 members: Ras1, Csc25, Srv1, Cyr1, Tpk1, Tpk2, Bcy1, Pde1, Pde2. and Efg1. As is common with many other *C. albicans* core signaling pathways, much of what we know about this pathway comes from the extensive study of the orthologous pathway in *S.* cerevisiae. *ScRAS1* (*S. cerevisiae RAS1*) and *ScRAS2* actually both correspond to *CaRAS1*. *CaRAS2* is a highly divergent ras protein with no *S. cerevisiae* orthologue. *CSC25* is the homolog of *ScCDC25.* Both *ScCYR1* and *CaCYR1* have until recently been known as CDC35, a similar situation exists for

CaSRV1 which has mostly been referred to *CAP1*, and so a great deal of the published literature refers to them as such.

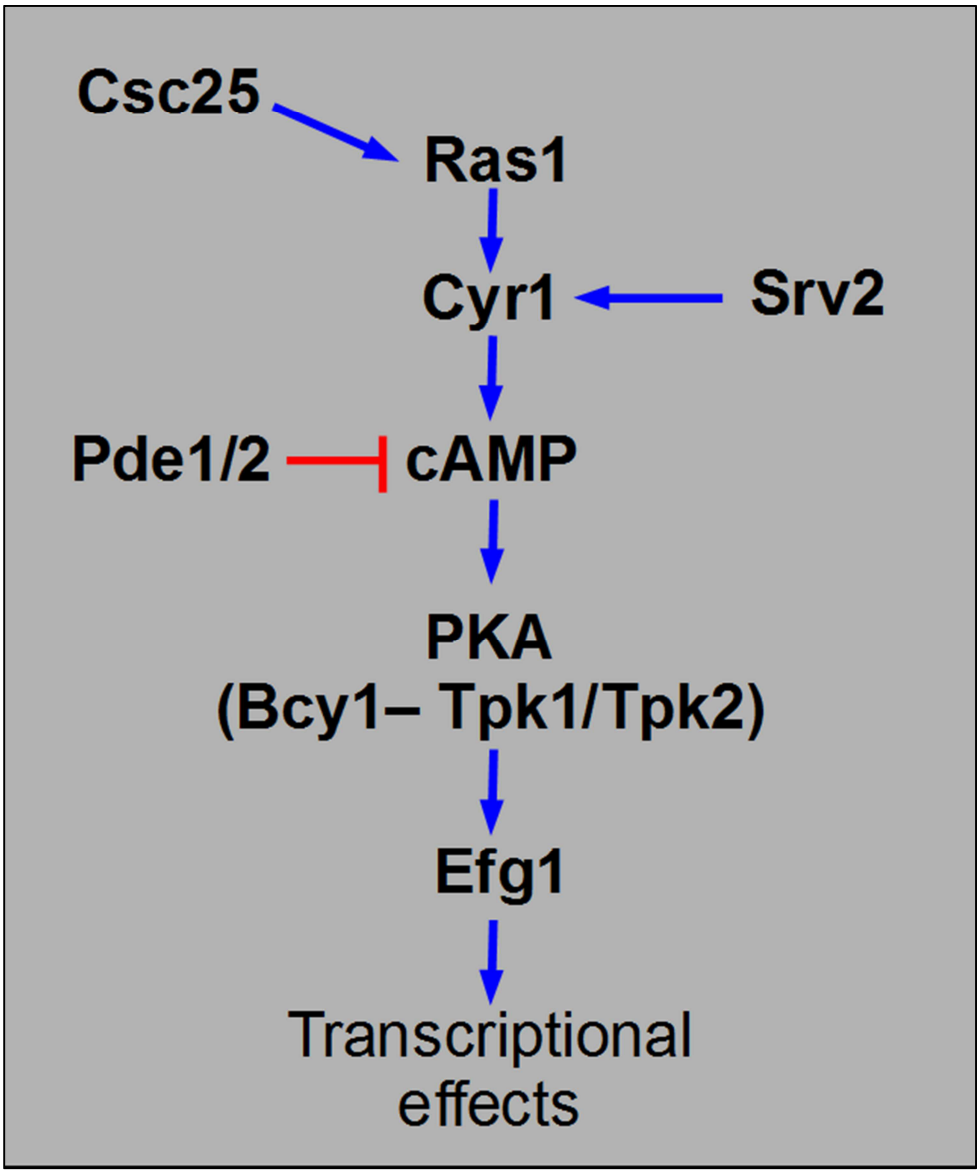

Figure 1-2: Overview of the Adenylyl cyclase – cAMP Pathway The sole fungal adenylyl cyclase, Cyr1, produces cAMP to influence the activity of the Protein Kinase A (PKA) complex. It is thought that PKA modulates the activity of the ASPES transcription factor Efg1 through phosphorylation, and that this promotes hyphal growth.

Our current understanding of the *C. albicans* cAMP pathway indicates that the basic function of each protein and the specific protein-protein interactions of pathway members with

each other are very similar to those in *S. cerevisiae*. Namely, *CaRAS1* encodes a small GTPase whose GTP-bound form can interact with and increase the specific activity of the sole fungal adenylyl cyclase, encoded by *CYR1*. *CSC25* encodes Ras1p's GEF. *SRV1* encodes the "cyclase associated protein" which binds Cyr1 at a domain far from the Ras1 binding site and activates Cyr1 (Zou *et al.*, 2010). cAMP levels are tightly tied to progression through the cell cycle in yeasts. *S. cerevisiae* is known to arrest in G1 phase if *CYR1* is deleted, making this gene essential unless downstream or parallel signaling components are overexpressed (Toda *et al.*, 1988).

Though Cyr1 and cAMP certainly play important roles in cell-cycle progression, *C. albicans* utilizes this pathway to sense and respond to hyphal inducers. It has been found that Cyr1, the sole fungal adenylyl cyclase, is an integrating center for temperature sensing, CO_2 levels, and the presence of MDPs (Muramyl-dipeptides, bacterial cell-wall breakdown products). Though temperature sensing is thought to involve Hsp90 (Shapiro *et al.*, 2009), *C. albicans* Cyr1 senses CO_2 and MDPs directly. Cyr1 functions as a homodimer. It is activated by environmental CO_2 when one copy of the protein binds bicarbonate ion (hydrated CO_2) through its catalytic Lysine. This increases the specific activity of the other catalytic subunit, thus leading to increased cAMP output (Hall *et al.*, 2010). MDPs were shown to be directly bound by the Cyr1 Leucine-rich repeat (LRR) domain, and their binding was shown to increase its specific activity (Xu *et al.*, 2008).

The cAMP produced by Cyr1p activates the Protein Kinase A complex by causing dissociation of the negative regulatory subunit, Bcy1, from the catalytic subunits, Tpk1 and 2 . Cellular cAMP-phosphodiesterases encoded by *PDE1* and *PDE2* act to downregulate cAMP activation of PKA by hydrolyzing cAMP, thereby lowering intracellular level. The balance

between production and hydrolysis of cAMP determines the intracellular level and thus the activation state of PKA. PKA can phosphorylate and modulate the activity of Efg1 transcription factor, which will be discussed later (Bockmuhl & Ernst, 2001).

The earliest published indication of the involvement of the cAMP pathway in the hyphal growth of *C. albicans* came in the form of an observation that induction of hyphal growth using a temperature shift caused an increase in the cellular levels of cAMP to just below two-fold the starting value (Niimi *et al.*, 1980). The increase in cAMP preceded the induction of hyphal growth by 30 minutes, suggesting it may be causal. This corollary relationship was specific to cAMP as the investigators found that cGMP levels remained constant. Additionally, the increase in cAMP and hyphal induction could be stopped simultaneously when adding excess cysteine to the media, again suggesting but not proving causation. Lastly the group was able to show that addition of dibutyryl-cAMP (db-cAMP), a cell permeable cAMP analogue, was able to enhance induction of hyphal growth under the conditions they employed. Using their culture media, which were typical of earlier *C. albicans* research but are no longer commonly employed, they were not able to show that addition of cAMP or its analogs could cause a switch to hyphal growth under conditions that would otherwise favor budding growth; this was a key piece of information that would come later.

Sabie and Gadd found that incubation with exogenous cAMP or db-cAMP could cause hyphal growth even in conditions lower than physiological temperature, which is normally a requirement for hyphal growth (Sabie & Gadd, 1992). This was a key piece of evidence and further suggested that the rise in intracellular cAMP preceding hyphal growth could be causal. Adding additional support, they also showed that treatments expected to increase intracellular

cAMP, namely, treatment with the cAMP-phosphodiesterase inhibitors caffeine and theophylline, were also able to cause a switch to hyphal growth.

Genetic approaches have yielded further evidence. Deletion of the high-affinity cAMP phosphodisesterase encoded by *PDE2*, predicted to cause higher than normal intracellular cAMP levels, was found to cause strains to be hyperfilamentous, that is, displaying pseudohyphal and hyphal growth in conditions where wild type would present as buds (Bahn et al., 2003). Expression of a constitutively active *RAS1* allele, *RAS1G13V*, or *CYR1* allele, *CYR1E1514K*, also caused hyperfilamentation (Davis-Hanna *et al.*, 2008, Bai *et al.*, 2011). Thus it is clear that an increase in intracellular cAMP, accomplished by either exogenous supplementation or genetic manipulation, can in and of itself effect hyphal growth in *C. albicans.*

The physiological rise in intracellular cAMP in response to hyphal inducers is different from those caused by exogenous supplementation with high levels of cAMP and expression of constitutively active cAMP pathway members. The physiological increase is transitory: cAMP levels reach a maximum of just about two-fold starting value 30-60 minutes after introduction to inducing conditions and then return to about 125% of the starting value for the duration of culture (Xu et al., 2008, Fang & Wang, 2006). Exogenous supplementation and constitutive Ras or Cyr1 would be expected to cause chronically high levels of cAMP. More direct evidence would be required to link the physiological rise in cAMP caused by inducers, termed a “spike”, to hyphal induction.

In *S. cerevisiae*, where a cAMP spike is caused by a sudden shift into dextrose containing media and plays a role in nutritional regulation, both Ras1 and its GEF Cdc25 are required for the spike (Gross *et al.*, 1999, Gross *et al.*, 1992). The study of a *Candida albicans*

RAS1 deletion mutant, and an insertional mutant of the *Candida albicans CDC25* orthologue *CSC25* showed that, like in *S. cerevisiae* glucose sensing, both are required for Cyr1 to effect a cAMP spike in response to serum, though the authors made no mention of the resulting effect on hyphal growth (Maidan *et al.*, 2005). Another study linked the necessity of the Cyr1 Ras-association domain (RA domain), which allows for Ras1 to bind and activate Cyr1, to both being required for a cAMP spike and required for hyphal growth in response to serum (Fang & Wang, 2006). The same group went on to show that sensing of MDPs through the LRR domain of Cyr1 causes a cAMP spike. Deletion of the LRR domain blocked MDP binding, a cAMP spike, and induction of hyphal growth (Xu et al., 2008). Thus a model began to emerge that signaling through adenylyl cyclase Cyr1 causes a cAMP spike, which induces hyphal growth and the HSGs.

Increased intracellular cAMP is expected to upregulate the Protein Kinase A complex by causing dissociation of the negative regulatory subunit Bcy1. Accordingly, overexpression of the PKA catalytic subunit encoded by *TPK2* caused strains to be hyperfilamentous on solid and liquid media, spontaneously developing hyphae and other filamentous growth forms (Sonneborn *et al.*, 2000). The link between PKA activity and hyphal induction is thought to come through its phosphorylation of the Efg1 transcription factor (Bockmuhl & Ernst, 2001).

Efg1 is an APSES-domain containing transcription factor. The domain is so named due to the names of the ascomycete orthologues; ASM-1, Phd1, StuA, Efg1, and Sok2. This family of fungal transcription factors has similarities in structure to bHLH (basic-helix-loop-helix) type transcription factors and are known to regulate metabolism, morphogenesis, and budding to hyphal transitions (Doedt *et al.*, 2004). Genetic evidence exists that PKA promotes hyphal growth solely through Efg1 (Mayer et al., 2013). As mentioned earlier, overexpression of PKA

catalytic subunit *TPK2* causes a hyperfilamentous phenotype on solid media. This was blocked however, when *TPK2* was overexpressed in an *EFG1* deletion mutant strain background (Mayer et al., 2013). A potential PKA phosphorylation site, Threonine-206, was found to modulate the hyphal promoting function of Efg1. Expression of *EFG1-T206A* blocked hyphal growth, while expression of the phosphomimetic *T206E* allele caused hyperfilamentation (Bockmuhl & Ernst, 2001).

1.5 A molecular mechanism for hyphal induction

Thus, a pathway from Adenylyl cyclase Cyr1, with its upstream activator Ras1, through cAMP to PKA and Efg1 has been mapped out. Genetic evidence existed that increased signaling at each of the levels could promote hyphal induction. A study by the Liu group proposed a model with molecular mechanisms that defined how increased signaling through the cAMP pathway could cause hyphal growth. It was proposed that hyphal growth was divided into two distinct phases; initiation and maintenance (Lu et al., 2011). The model was that an increase in temperature or washing out of hyphal-inhibiting quorum sensing factors, both reported to exert their action through the cAMP pathway, would cause an upregulation in signaling through the cAMP pathway and cause the ejection of the Nrg1 hyphal repressor protein from the promoters of HSGs. This caused entry into hyphal growth, that is, hyphal induction, and induced HSGs. This was defined as the initiation phase of hyphal growth.

The group further demonstrated that if another signal, in the form of a hyphal inducer, was not provided simultaneously or soon after the initial removal of Nrg1 from hyphal promoters, Nrg1 would re-localize back to hyphal promoters, downregulate HSGs, and that this corresponded with a switch back to budding growth. If however a hyphal inducer was added during the time Nrg1 was not present, the Hda1 deacetylase would be recruited instead. Hda1 is

thought to deacetylate the Yng2 subunit of the NuA4 histone acetyltransferase complex. Hda1 deacetylation of the Yng2/NuA4 complex leads to its ejection from the promoter, and reconfiguring of the promoter chromatin such that Nrg1 cannot return. Thus HSGs remain active and hyphal growth would continue for as long as there were nutrients and a hyphal inducer (Lu et al., 2011).

The mechanism of action of hyphal inducers causing relocalization of Hda1 to hyphal promoters was partially explained. Nutrient-poor hyphal inducing media such as SCAA, M199, and Lee's medium, were thought to cause a decrease in signaling through the nutrient sensitive TOR pathway, and that this signal was what caused relocalization of Hda1. In agreement with this it was shown that Rapamycin could cause relocaliztion of Hda1 to hyphal promoters, and cells with a hyperactive TOR pathway could not sustain hyphal growth on nutrient poor media. Though dependency on the TOR pathway was not addressed, the ability of GlcNAc and serum to recruit Hda1 to hyphal promoters was demonstrated (Lu et al., 2011).

The Initiation – Maintenance model centered around the hypothesis that exclusion of Nrg1 from the promoters of HSGs was able to cause hyphal growth. This seemed plausible because deletion of *NRG1* causes a strong hyperfilamentous phenotype (Murad et al., 2001). Indeed, the *NRG1* deletion mutant is often used as the standard for a constitutively filamentous, "hyphal-locked" strain when experiments call for such a strain. A weakness in the model was that Nrg1 is a transcription factor and its exclusion from hyphal gene promoters by deletion or other means was expected to cause hyphal growth through a change in gene regulation. However, no gene or gene set modulated by ejection or deletion of Nrg1 has been identified and associated with a mechanism for hyphal induction (Lu et al., 2011).

1.6 Blocking signaling through the cAMP pathway causes defects in the induction of hyphal growth

Along with the evidence that increased signaling through the cAMP pathway can promote or cause hyphal growth, there exists a large body of evidence that signaling through the cAMP is necessary for hyphal growth: Deletion of *RAS1* was reported to cause mild to severe defects in hyphal growth in both solid and liquid media (Leberer *et al.*, 2001), deletion of *TPK2* causes filamentous growth defects in liquid media (Sonneborn et al., 2000) and deletion of *EFG1* caused severe defects in liquid induction of hyphal growth (Bockmuhl & Ernst, 2001). The most severe phenotype however, was that of the adenylyl cyclase deletion mutant *cyr1Δ*.

cyr1Δ cells are misshapen, enlarged, and growth at about one third the rate of wild type cells (a 3 to 4 hour doubling time in rich YPD medium) (Rocha et al., 2001). As *CYR1* encodes the sole fungal adenylyl cyclase, *cyr1Δ* cells lack all detectable cAMP. Unlike the incomplete hyphal and filamentous growth defects found in other pathway members, *cyr1Δ* cells are completely defective in hyphal growth even under the strongest hyphal conditions used. This had led to the idea that signaling through Adenylyl cyclase is required for hyphal growth in *C. albicans*.

This hypothesis fits well with the Initiation – Maintenance model of hyphal growth; deletion of *CYR1* precludes a cAMP spike and ejection of Nrg1 from the promoters of hyphal gene, thereby blocking initiation of hyphal growth. It also predicts that an increase in cAMP in the *cyr1Δ* cells should be able to initiate hyphal growth. As will be discussed in Chapter 3, we have found that supplementation with high levels of exogenous db-cAMP can cause even *cyr1Δ* to filament.

It was found however that the Hda1 mediated deacetylation of Yng2/NuA4, though critical for hyphal maintenance *in vitro*, was not required for maintenance of *C. albicans* hyphae in an *in vivo* infection setting (Lu *et al.*, 2013). A key piece of evidence supporting that maintenance of hyphal growth was accomplished through the action of Hda1 in remodeling HSG promoters was that a strain expressing *YNG2K175Q* as the sole source of *YNG2* could not maintain hyphal growth past 5 hours *in vivo.* The K175Q substitution mimics acetylation and is also unable to be deacetylated by Hda1 and restricted from promoters, allowing for their reconfiguration and maintenance of hyphal growth. While this strain was unable to maintain hyphal growth under all *in vitro* conditions tested, it was fully virulent in the mouse model of disseminated candidiasis and was found to present as a mixture of hyphal, Pseudohyphae, and buds in infected organs similar to wild type (Lu et al., 2011, Lu et al., 2013).

The study went on to implicate CO_2 and hypoxia, conditions that are absent from most *in vitro* hyphal induction experiments, as acting through alternate pathways to directly stabilize the pro-hyphal transcription factor Ume6. *UME6* encodes a pro-hyphal Zn(II)2Cys6 transcription factor. It was recently demonstrated that overexpression of *UME6* from a regulatable promoter was sufficient to cause hyphal growth(Carlisle & Kadosh, 2013). The pathway through which CO_2 influenced the stability of Ume6 was not discussed though it was demonstrated that Ofd1, a prolyl 4-hydroxylase-like 2-oxoglutarate-Fe(II) dioxygenase, regulates Ume6 turnover (Lu et al., 2013). Ofd1 is suspected to posttranslationally hydroxylate Ume6, which promotes its degradation. Orthologues of this enzyme are sensitive to oxygen levels and deactivated in hypoxia, thus, hypoxia allows accumulation of Ume6. Abrogating both the stabilization of Ume6 and the Hda1-mediated hyphal maintenance pathway caused virulence to be attenuated but did not prevent filamentous growth *in vivo (Lu et al., 2013).*

1.7 Inadequacies of the current model

The current model of hyphal induction through the action of transcription factors has not provided a mechanism for how the stabilization of Ume6 or downregulation of *NRG1* could cause hyphal growth. Many of the genes regulated by Nrg1 and Ume6 are HSGs and, as described earlier, the only HSG required for hyphal growth is *HGC1*. Though *HGC1* is required for hyphal growth, overexpression of *HGC1* is not sufficient to cause hyphal growth (Zheng & Wang, 2004). Additionally, though *HGC1* deletion cells are unable to support true hyphal growth, they initiate a form of filamentous polarized growth that quickly deteriorates into isotropic budlike growth. This indicates that though Hgc1 is required for hyphal growth, it is not required for earlier events of the bud-hyphal decision and initial polarized growth. Importantly, under certain conditions hyphal induction can be accomplished without induction of HSGs, further challenging the model that HSG induction causes hyphal induction (Naseem *et al.*, 2015).

There are also indications that the hyphal growth caused by overexpression of *UME6* or deletion of *NRG1* is by non-physiological means. The *NRG1* deletion mutant was found to have transcriptionally upregulated the signaling mucin *MSB2* 3.9 fold and the MAP kinase *CEK1* 4.9 fold (Zordan *et al.*, 2007). *MSB2* is a transmembrane mucin that can signal through the MAP kinase pathway in both *S. cerevisiae* and *C. albicans* (Roman *et al.*, 2009, Jin *et al.*, 1995). Importantly, overexpression of *MSB2* alone in *S. cerevisiae* causes hyperfilamentation, with cells presenting as pseudohyphae even under normal culture conditions (Jin et al., 1995). Neither of these genes was found to be upregulated in cells induced to form hyphae in serum or GlcNAc (Nantel et al., 2002, Gunasekera et al., 2010). Thus the hyperfilamentation seen in *NRG1* deletion mutants may be due to upregulation of two components of the MAP Kinase pathway, which are not upregulated during treatment with the physiological hyphal inducers GlcNAc and

serum. Likewise, overexpression of *UME6* caused a 3.7 fold upregulation of *CEK1*, though no change in *MSB2* was detected *(Lan et al., 2002)*. That hyphal growth could be caused by a 3.7 fold increase in one MAP Kinase component may seem doubtful, but it should be noted that the filamentous growth evolving from *UME6* overexpression is 80% pseudohyphal by 3 hours and 100% hyphal by 10 hours, as opposed to the nearly 100% hyphal filamentation seen 90 minutes after serum induction ((Lan et al., 2002) and our unpublished observations).

The model of induction and maintenance of hyphal growth through induction of HSGs has been challenged significantly by recent findings. Key to this model is that signaling through Adenylyl cyclase and an increase in cAMP are thought to be required for hyphal initiation. The evidence supporting this requirement was that *cyr1Δ* cells fail to induce hyphal growth and hyphal genes (Rocha et al., 2001, Lu et al., 2011, Gunasekera et al., 2010). Interpretation of the *cyr1Δ* mutant phenotype, and indeed all cAMP pathway deletion mutant phenotypes, is complicated by the negative impact on cellular health associated with perturbation of this pathway. In an effort to better define the role of the cAMP pathway in hyphal growth we undertook the study detailed in Chapter 3. We have data that suggest the hyphal defects of cAMP pathway member deletion mutants are due to secondary effects of on cellular health, and not to a specific defect in hyphal signaling. Also, using *cyr1Δ* pseudorevertants, we demonstrate that neither adenylyl cyclase Cyr1 nor cAMP are required for hyphal growth in response to various physiological inducers. Taken together this strongly suggests that cAMP – independent pathways make an important contribution to hyphal growth in *C. albicans*.

Chapter 2: Materials and Methods

Strains and media

The *C. albicans* strains used in this study are described in **Chapter 3 Table 1**. *C. albicans* cells were grown in rich yeast extract-peptone-dextrose (YPD) medium or in complete synthetic medium (SC) made with yeast nitrogen base (YNB) and select amino acids or uridine (Uri) (Sherman, 2002). Homozygous gene deletion mutant strains were constructed either in strain BWP17 URA3+ or in the indicated parental strain by the sequential deletion of both copies of the targeted gene (*C. albicans* is diploid). BWP17 URA3+ was generated by transforming BWP17 (Wilson *et al.*, 1999) with a *URA3*-containing fragment to complement the auxotrophy. The plasmid pBSK-*URA3* was digested with the restriction enzymes Pst1 and Not1 to liberate the *URA3-IRO1* sequence, which was then transformed into BWP17 and integrated by homologous recombination into the genome to restore *URA3* at its native locus as well as the fragment of the IRO1 gene removed during the making of BWP17.

The *csc25Δ* (SP53-1), *ras1Δ* (SP54-1), and *cyr1Δ* (SP57-1) strains were constructed by deleting one copy of the indicated gene using the *SAT1* flipper (Reuss *et al.*, 2004). The *SAT1* flipper was PCR amplified in a manner that added 80 bp on each end of DNA sequence that flanked the open reading frame of the targeted gene to promote homologous recombination. The *SAT1* flipper contains a nourseothricin resistance marker *CaSAT1*, and a modified flippase gene, *CaFLP*. The appropriate mutants were identified by PCR analysis using a combination of primers outside the sites of cassette integration and internal primers. The second allele was deleted in a similar manner using a PCR amplified version of the *ARG4* selectable marker with 80 bp of flanking homology to the targeted gene. The histidine auxotrophy was corrected either with a PCR product containing 2050 bp of *HIS1* or a *GFP-HIS1* cassette (Zhang & Konopka, 2010) targeted to the *HWP1* locus to create the *HWP1-GFP* reporter gene in strains SP57-1 and

SP62-88. Gene deletion was verified by PCR analysis using a combination of primers that detect the gene deletion cassettes at the locus as well as primers designed to detect the open reading frame. Strain PR1-*wor1Δ* was constructed from strain PR1 by sequential deletion of both copies of *WOR1* using the *SAT1* flipper method (Reuss et al., 2004).

Hyphal growth assays

Unless otherwise indicated, cells were grown overnight at 30°C at low density ($<1x10^7$ cells ml^{-1}) in SC-Ura medium lacking uracil and containing 100 mM dextrose. The following morning, 1.5 ml aliquots were harvested by centrifugation, washed with 1 ml of YNB, inoculated into 7 ml of SC-Ura + 100 mM dextrose or 100 mM GlcNAc medium, and incubated at 37°C for the indicated time to induce hyphal morphogenesis. Serum induction was performed by inoculating cells into SC-Ura medium containing 100 mM dextrose and 30% Bovine Calf serum. Alkaline pH induction of hyphal growth was performed by modification of a previously described method (Murad et al., 2001) in which cells were cultured overnight in YPD at 30°C, washed, and then inoculated into SC containing 0.1% dextrose + 150 mM HEPES buffered to pH 8, and cultured overnight at 37°C before visualization. CO_2 induction was performed by a modification of a previously described protocol (Klengel et al., 2005). Cells were inoculated into YNB medium containing 100 mM dextrose and 150 mM PIPES buffered to pH 7 in a total volume of 100 µl in a 96-well plate. The plate was covered with a breathable cover and incubated overnight at 37°C with 5% CO_2, and then the next day the cells were removed from the wells and examined by microscopy.

To assess the production of GFP by the *HWP1*-GFP reporter gene, a hyphal growth induction was performed with the GFP-tagged strains and then live cells were harvested and examined using a fluorescence microscope.

Staining cells with Hoechst 33342 and Pontamine Fast Scarlet 4B

To visualize cell septae and nuclear DNA, cells were first stained with Hoechst 33342 (2 µg ml^{-1}; Invitrogen, Grand Island, NY) to detect DNA and then stained with Pontamine Fast Scarlet 4B (0.3 µg ml^{-1}; gift from Charles Sprecht, University of Massachusetts Medical School, Worcester, MA) to detect cell wall and septae. Pontamine Fast Scarlet 4B stains cells similar to Calcofluor White (Hoch *et al.*, 2005) but it fluoresces at a distinct wavelength from Calcofluor White, making it possible to carry out double-staining analyses with Hoechst stain.

db-cAMP supplementation

Cells of strain CR216 *cyr1Δ* were grown overnight in YP medium containing 100 mM galactose. Galactose was used because, unlike dextrose, it does not repress the expression of the GlcNAc specific transporter *NGT1* (Alvarez & Konopka, 2007). The next day, cells were washed and resuspended in the same medium with the indicated amount of db-cAMP at a density of 5 x 10^6 cells ml^{-1} in a total volume of 150 µl in a 96-well microplate with an air permeable cover. The cells were cultured for 2 hours at 37°C before addition of GlcNAc, then grown an additional 3 hours. Cells were imaged in the microplate. Quantification was performed by counting at least 300 cells per experiment in 3 separate experiments.

Pseudorevertant frequency assay

Strain CR216 was freshly streaked from a frozen stock and grown on YPD plates at 30°C. Four different colonies were then restreaked onto fresh plates and grown for 4 days at 30°C. The plates were then incubated at room temperature for 1, 2, 3, or 4 weeks. After incubation at room

temperature, 32 different colonies were restreaked on YPD and incubated at 30˚C. The number of large and small colonies was then counted. This assay was performed three independent times. Similar results were observed in similar experiments with *cyr1Δ* strain SP60-66.

Intracellular cAMP determination

cAMP was extracted using the protocol essentially as described previously (Davis-Hanna et al., 2008). In the case of basal cAMP level measurements, overnight dilution series consisting of 4 culture tubes per strain were set up. The first tube contained 5 ml while the other 3 contained 7 ml of media before dilution. For wild type DIC185, inoculating the first culture tube with a small amount of cells off of a fresh plate, a spherical cell clump approximately 3 times the diameter of the periods used at the end of the sentences in this document, and then performing 1 ml serial transfers through the rest of the 3 culture tubes, resulted in the 3rd or 4th tube having between 0.5 – 3 x10^{8} cells/ml after an overnight incubation on a roller at 30°C.

note For pseudorevertant strains approximately 1.5 times the amount of cells should be used for this culture routine, while non-pseudorevertant cyr1Δ strains require approximately 4 times the amount of cells and 2 ml serial transfers through the culture tubes to achieve similar growth by the next day.*

The next day, the culture dilutions closest to 0.5 – 3 x10^{8} cells/ml were chosen, and 1x10^{8} cells were pelleted in standard 1.5 ml microfuge tubes for 30 seconds at max speed in a standard microcentrifuge and all of the supernatant carefully removed.

note Generally, no more than 30 seconds at the max speed of normal microcentrifuges is required for pelleting C. albicans. If more than 1.5 ml was required to achieve the correct*

number of cells, 1.5 ml of culture would be spun down first, the supernatant removed, and the remaining required volume added and spun down again.

Once a pellet of $1x10^8$ cells was achieved for all strains, the pellets were snap frozen in liquid nitrogen by dropping them into 500 mL of liquid nitrogen. It should be noted that the time between centrifugation of the culture and snap freezing should be minimized as much as possible. After allowing the pellets to freeze in liquid nitrogen for 10 minutes, the tubes were pulled out, thrust into a full ice-bucket, and the tops opened carefully to allow the tubes to remain on ice but to avoid any ice falling into the tubes. The cAMP extraction was begun by adding 1 ml of ice-cold 1 M Formic acid saturated with N-butanol to each of the tubes.

note This solution was prepared beforehand in 50 ml conical tubes with approximately 35 ml of the 1 M Formic acid solution being overlaid with 10 mL N-butanol, then vortexed for 10 seconds and allowed to settle for a half hour. This solution can be prepared days ahead of time, though preparations more than a few weeks old were never tested for efficacy. On the day of the experiment, the Formic Acid solution should be allowed to cool on ice for at least an hour before use, to ensure it is truly ice-cold.*

The samples were then vortexed at top speed, one at a time, for at least 10 seconds or more until the cell pellet is completely resuspended. Samples were kept on ice and mixed 10 seconds each, 3 more times, over the course of 10 minutes. After this, cell debris were pelleted in a 4°C refrigerated microcentrifuge at 20,000 x g for 10 minutes. 150 µl of the supernatant was collected in a separate tube and dried under high vacuum with heating.

note Once the supernatant is collected it is no longer necessary to maintain the samples on ice. As the samples heat up some or all of them may begin to appear cloudy; this is normal and had no discernable effect on the experiment. In practice, up to 750 ul of supernatant can be*

easily collected without disturbing the cell debris. This can allow for a single sample to be assayed multiple times, but using a cAMP pellet from a volume greater than 150 ul caused an increase in background signal when assayed. Thus, a cAMP pellet of 150 ul extract represents the optimum amount for use in this assay. Additionally, the rate of evaporation is incredibly slow: even under high vacuum at 45°C 150 ul samples may take between 4-12 hours to completely evaporate while 750 ul samples may require more than 24 hours. It is critical that a vacuum centrifuge with a condenser apparatus is used, as the Formic Acid will destroy vacuum pumps. The Reich lab at Stony Brook University, graciously allowed us to use their vacuum setup for the preparation of the cAMP samples assayed in Chapter 3. Once cAMP extracts are dried down to pellets, they can be stored at -80°C until they are ready to be used. It should also be noted that little or no visible material will be present in the cAMP "pellets"; this is normal as the amount of cAMP extracted is actually quite small. Sometimes a thin, clear, solid substance approximately 2-3 mm in diameter can be found after drying. I suspect that this is leftover Dextrose that has recrystallized during drying; its presence or absence had no discernable effect on experimentally measured values, but tubes that manifested it required significantly more drying time than those that did not.

The amount of cAMP was determined using a cAMP Direct Biotrak EIA immunoassay kit (GE Amersham, Pittsburgh, PA). The dried cAMP pellet was resuspended in 1 ml of sample buffer and assayed using either the non-acetylation or acetylation protocols as per the manufacturer's instructions.

note GE Amersham changed the reagents in their kit recently: this has caused the Non-acetylation assay to be too insensitive to be used for our purposes. Thus, the Acetylation protocol should be used when assaying cAMP levels. No deviations were made from the*

protocol as written in the kit manual, and cAMP pellets are still diluted to 1 mL in cAMP assay buffer as described. The acetylation reagent can be prepared, as per the instructions in the manual, in any small glass tube but the 3-5 ml glass tubes with tops from the Del Poeta lab at Stony Brook proved to be the most convenient as the mixture is volatile and the vapors irritating.

For determination of cAMP levels in strains treated with Serum as a hyphal inducer, the extraction steps were identical starting from the point of having obtained a snap-frozen pellet of 1 x10^8 cells in a 1.5 ml microfuge tube; the culture conditions were different however.

An identical dilution series was performed for the overnight culture, with the exception that the media used was SC-URA + 100 mM Dextrose, and approximately twice the amount of cells were used for the inoculation of the first tube as strains grow more slowly in defined medium than in rich YPD. After overnight incubation at 30°C cultures of densities between 0.5 – 3 x10^8 cells/ml were selected and 1x10^8 cells were pelleted as described before. Once the pellets were obtained in the microfuge tubes, they were washed once with 1 ml BYNB, and resuspended in 1 ml SC-URA 100 mM Dex + 30% Bovine Calf Serum pre-warmed to 37°C. The tubes were then transferred to the 37°C in their normal microfuge tube racks and allowed to incubate for 30 minutes. After incubation, the cells were pelleted, the supernatant carefully removed, and snap frozen in liquid nitrogen. The rest of the cAMP extraction steps were identical to those described before. The amount of cAMP was determined using the Acetylation protocol of the aforementioned kit.

Chapter 3: cAMP-independent signal pathways promote hyphal induction of *Candida albicans*

3.1 Abstract

The human fungal pathogen *Candida albicans* undergoes a transition from budding to hyphal growth in the host that promotes invasive growth into tissues. Previous studies demonstrated that stimulation of adenylyl cyclase to form cAMP induces hyphal morphogenesis and gene expression. Accordingly, the failure of various hyphal inducers to stimulate cells lacking adenylyl cyclase (*cyr1Δ*) has suggested that most inducers act through cAMP induction. However, *cyr1Δ* mutants also grow slowly and have defects in morphogenesis. It was therefore unclear whether all inducers must act by stimulating cAMP production, or if a normal basal level of cAMP is required to maintain cellular health and permit hyphal growth. To clarify the role of cAMP, we supplemented a *cyr1Δ* mutant with low levels of exogenous cAMP and found that this allowed the cells respond to the hyphal inducer N-acetylglucosamine (GlcNAc), indicating that a basal level of cAMP is sufficient for stimulation. Furthermore, we isolated *cyr1Δ* pseudorevertant strains that grow better and can be induced to form hyphae in response to serum, GlcNAc, and alkaline pH. The pseudorevertants were not induced by CO_2, consistent with reports that CO_2 directly stimulates adenylyl cyclase. Thus, cAMP-independent signals contribute to the induction of hyphal responses.

3.2 Introduction:

Candida albicans is a human fungal pathogen that is commonly found in the GI tract as a commensal organism (Odds, 1988, Heitman et al., 2006). However, severe systemic infections can result from conditions that promote the overgrowth of *C. albicans* in the host, or compromise the immune system. Better therapies are needed to treat systemic candidiasis, as even with advances in current antifungal therapy there is about 40% attributable mortality (Pfaller & Diekema, 2010). A key factor for virulence is the ability of *C. albicans* to undergo a transition from growing as budding cells to instead forming long chains of hyphal or pseudohyphal cells that grow invasively into tissues (Sudbery, 2011). This change in the pattern of morphogenesis to filamentous growth is also important for *C. albicans* to form biofilms on catheters and medical devices (Blankenship & Mitchell, 2006). Cells induced to form hyphae also show increased expression of virulence factors including adhesion proteins that promote biofilm formation and enzymes that protect the cells from oxidative attack by the immune system (Whiteway & Oberholzer, 2004, Kumamoto & Vinces, 2005, Blankenship & Mitchell, 2006).

A variety of different stimuli induce *C. albicans* to undergo hyphal growth *in vitro* including serum, alkaline pH, CO_2, bacterial peptidoglycan breakdown products, and N-acetylglucosamine (GlcNAc) (Biswas *et al.*, 2007, Whiteway & Bachewich, 2007, Davis, 2009, Sudbery, 2011). Several signal transduction pathways contribute to induction of hyphal growth in *C. albicans*; however, the cAMP pathway has been considered the most important (Wang, 2013, Whistler & Rine, 1997). One reason for this is that cells lacking the sole *C. albicans* adenylyl cyclase, Cyr1, are devoid of cAMP and do not respond to a wide range of hyphal inducers (Rocha et al., 2001). In addition, Cyr1 can be activated by several mechanisms and is therefore thought to act as a key sensor that integrates information from the environment. Cyr1

is activated by the small GTP-binding protein Ras1, the Gα protein Gpa2, and it can be directly activated by peptidoglycan breakdown products or bicarbonate derived from CO_2 (Wang, 2013, Whistler & Rine, 1997).

Data supporting a key role for adenylyl cyclase in hyphal induction include the observation that addition of exogenous cAMP can stimulate hyphal growth (Rocha et al., 2001, Sabie & Gadd, 1992). Furthermore, mutants with higher basal levels of cAMP are hyperfilamentous, such as a mutant lacking the Pde2 cAMP phosphodiesterase or cells that produce constitutively active forms of Ras1 or Cyr1 (Bahn et al., 2003, Davis-Hanna et al., 2008, Bai et al., 2011). However, sustained elevation of cAMP levels do not appear to be necessary to maintain hyphal growth, since hyphal inducers have been reported to cause only a transient spike in cAMP levels (Maidan et al., 2005, Fang & Wang, 2006, Toda et al., 1988).

Stimulation of Cyr1 results in the activation of the PKA kinase and subsequent induction of hyphal morphogenesis (Maidan et al., 2005, Fang & Wang, 2006, Toda et al., 1988). The events downstream of PKA are not well understood, but hyphal morphogenesis is regulated in part by activation of the Hgc1-Cdc28 cyclin-dependent kinase complex and phosphorylation of morphogenesis proteins (Sudbery, 2011, Mayer Fç *et al.*, 2013). Induction of hyphal-specific genes that are regulated by the Efg1 transcription factor (Toda et al., 1988) is thought to promote hyphal morphogenesis, but other pathways are also thought to exist (Naseem et al., 2015, Kronstad *et al.*, 2011, Zucchi *et al.*, 2010).

The failure of the *cyr1Δ* mutants lacking adenylyl cyclase to respond to a wide variety of hyphal inducers has led to the interpretation that cAMP signaling is necessary for most types of hyphal growth (Wang, 2013, Whistler & Rine, 1997). However, the *cyr1Δ* mutant also has other phenotypes that likely contribute to its defect in hyphal morphogenesis. For example, the *cyr1Δ*

mutant grows about three-fold slower than wild type cells, presumably due to altered expression of metabolic genes (Rocha et al., 2001, Harcus *et al.*, 2004). The Cyr1 protein also complexes with actin and Srv2 in wild type cells, indicating it may have other roles in morphogenesis in addition to the production of cAMP (Zou et al., 2010). Thus, it is not clear that all inducers specifically act through cAMP, or if a normal basal level of cAMP is needed for cells to be competent to respond to hyphal inducers. To better define the role of cAMP signaling in *C. albicans* hyphal growth, we showed that supplementing *cyr1Δ* cells with low levels of a cell permeable cAMP analog enabled them to induce hyphal growth in response to GlcNAc. Additionally, we discovered spontaneous pseudorevertants of *cyr1Δ* that grew at near wild type rate and induced rapid hyphal growth. These results indicate that cAMP-independent pathways also promote the transition to hyphal growth.

3.3 Results

3.31 Role of Csc25, Ras1, and Cyr1 in hyphal induction

To initiate an analysis of the cAMP pathway in inducing *C. albicans* hyphal growth, we examined the effects of deleting *CYR1*, *RAS1*, and *CSC25*, which encode adenylyl cyclase, its upstream activator *RAS1*, and *CSC25*, which is the closest homolog of the *S. cerevisiae CDC25* that encodes a guanine nucleotide exchange factor for Ras1. Although *cyr1Δ* and *ras1Δ* deletion mutants have been studied, to our knowledge only an insertional mutation of *CSC25* was analyzed in previous studies (Enloe *et al.*, 2000, Maidan et al., 2005). Therefore, we compared the effects of deleting these genes in a similar manner in the same strain background. The mutant cells were then induced with GlcNAc to determine if they could undergo hyphal morphogenesis. Wild type cells induced in GlcNAc medium showed the expected formation of small hyphal outgrowths (germ tubes) by 90 min (Fig. 1). In contrast, the *csc25Δ* mutant showed limited germ tube formation at 90 min, and the *ras1Δ* and the *cyr1Δ* mutants showed strong defects in filamentous growth, similar to results for the previously described for strain *cyr1Δ* CR216 (Rocha et al., 2001).

After 5 h incubation, wild type cells formed elongated chains of hyphal cells (Fig. 1). Interestingly, the *csc25Δ* cells also formed hyphae, indicating they could do so after a lag. The *ras1Δ* mutant also formed some filamentous cells after 5 h, but to a lesser degree. The *cyr1Δ* mutant strains failed to induce any filamentous growth forms even after 5 h. Filamentation at 5 h was not due to the effect of GlcNAc catabolism on extracellular pH, as using media buffered to pH 4 did not prevent filamentation. Previous studies have shown that growth of cells on GlcNAc medium raises the extracellular pH, which can also contribute to hyphal signaling, presumably because excess nitrogen is exported as ammonia (Naseem et al., 2015).

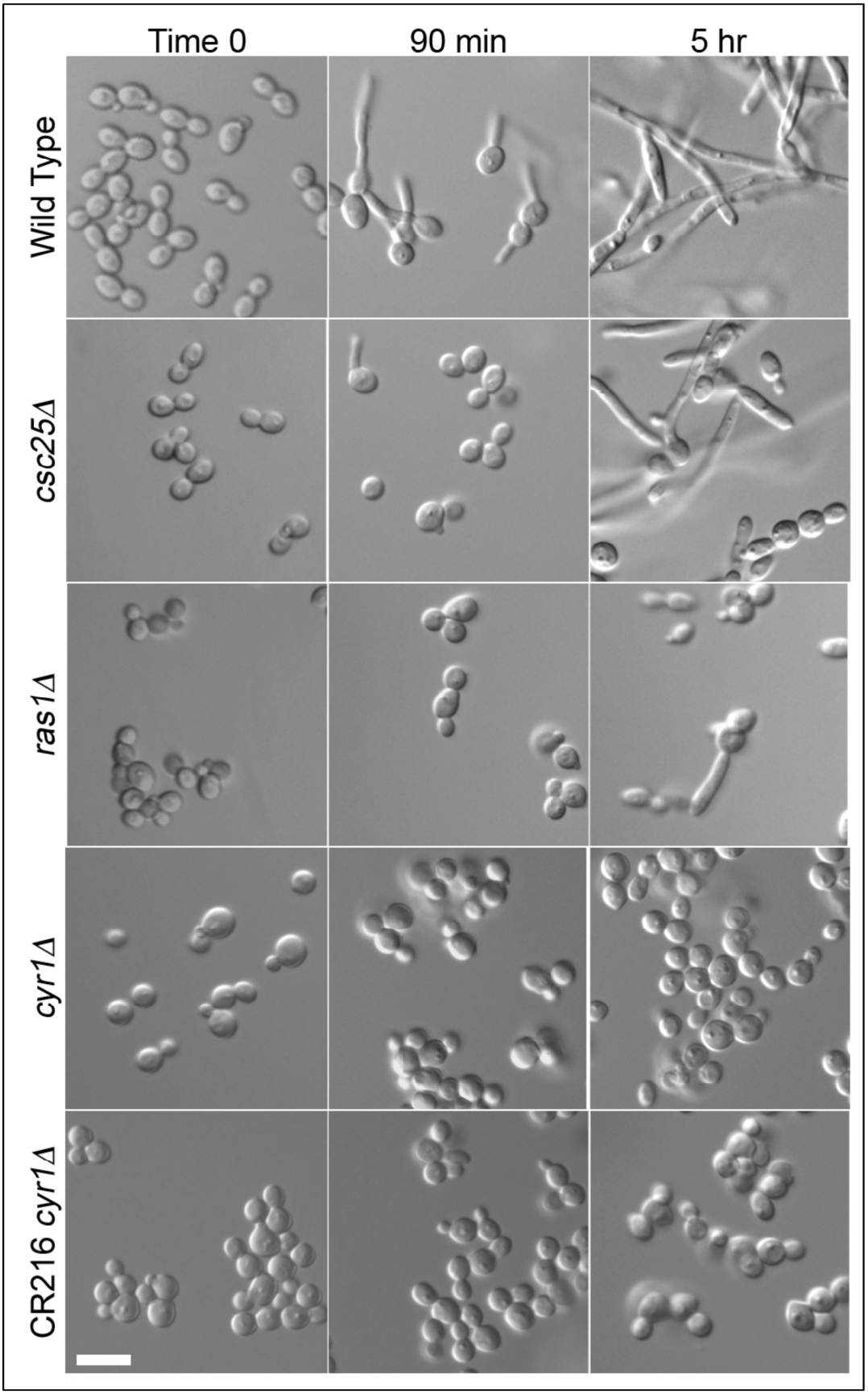
Time 0
90 min
5 hr
Wild Type
csc25Δ
ras1Δ
cyr1Δ
CR216 cyr1Δ

Figure 1. Deletion of cAMP pathway members results in hyphal defects.

Hyphal formation was induced by growing cells in synthetic medium containing 100 mM GlcNAc at 37°C for the indicated time before being pelleted and analyzed using DIC microscopy. The wild type (DIC185), *csc25Δ* (SP53-1), *ras1Δ* (SP54-1), *cyr1Δ* (SP60-66) strains were derived from BWP17 and the CR216 *cyr1Δ* strain was derived from the CAI4 strain of *C. albicans* as described previously (Rocha et al., 2001). Bars, 10 µm.

3.32 Synergy between GlcNAc and exogenously added cAMP

To more directly examine if it is a sudden rise in cAMP or just a normal basal level of cAMP that is important for hyphal growth, we restored intracellular cAMP levels by supplementing *cyr1Δ* cells with the cell permeable cAMP analog dibutyryl-cAMP (db-cAMP). Specifically, we cultured cells of strain CR216 *cyr1Δ* in a 96-well plate with various concentrations of db-cAMP. The cells were grown in medium containing galactose as a nutrient source, because it does not repress the expression of the GlcNAc-specific transporter Ngt1 as does glucose, thus allowing cells to grow normally and remain fully able to respond to GlcNAc (Alvarez & Konopka, 2007). After an initial incubation for 2 h at 37˚ C, GlcNAc was added to a final concentration of 10 mM to one set of cultures and then cells were incubated for an additional 3 h. In the absence of GlcNAc, filamentous growth was obvious at db-cAMP concentrations of 10 and 20 mM, but not at the lower concentrations (Figure 2A, top row). In contrast, GlcNAc induction of filamentous growth was detectable at the lowest concentration of db-cAMP tested (1 mM) and became more prevalent at higher concentrations of db-cAMP (Figure 2A, bottom row). This suggests that neither adenylyl cyclase Cyr1 nor a cAMP spike are necessary for GlcNAc-induced filamentous growth, and that it is a basal level of cAMP that is important for cells to be able to respond.

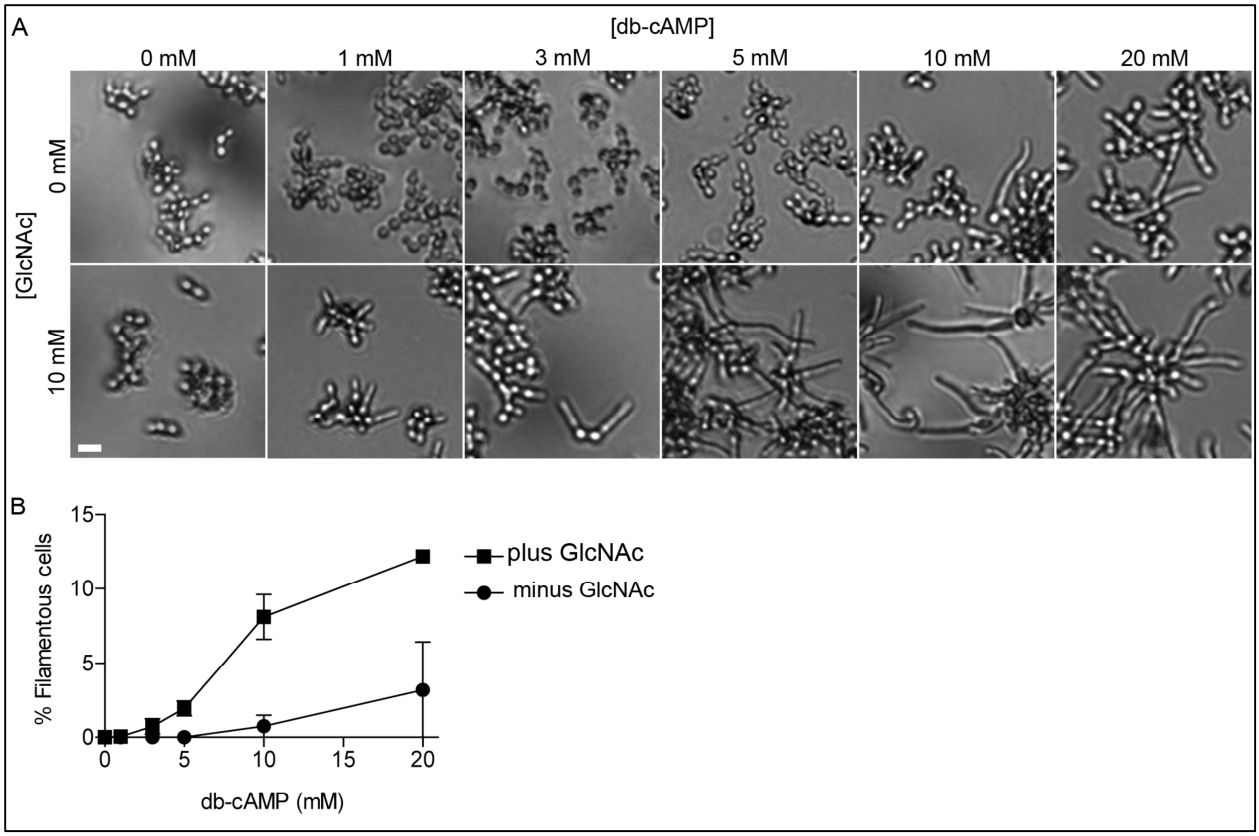

Figure 2. Addition of non-inducing levels of db-cAMP to *cyr1Δ* restores hyphal switching in response to GlcNAc.

A. Cells of strain *cyr1Δ* strain CR216 were cultured in rich YP medium containing 100 mM galactose plus the indicated amount of dibutyryl cAMP (db-cAMP). The cells were incubated at 37°C for 2 h, GlcNAc was added to a final concentration of 10 mM, and then the cells were incubated at 37°C for 3 h before being photographed. Bars, 10 µm.

B. Quantification of the average number of filamentous cells induced in the experiments described in panel A. Pre-incubation of *cyr1Δ* cells with non-inducing concentrations of db-cAMP restored the ability of GlcNAc to stimulate hyphal growth. The results represent the average of three different experiments, with at least 300 cells were counted per experiment. Error bars indicate SD.

3.33 Pseudorevertants of *cyr1Δ* exhibit better growth and can be induced to form hyphae

During the course of studying *cyr1Δ* mutants we noticed that cells that had been maintained for a few weeks on agar medium exhibited two populations of cells when restreaked onto a fresh agar plate; the expected small colonies and also large colonies that grew similar to a wild type control (Fig. 3A). Further analysis showed that the larger colonies arose in a time-dependent manner (Fig. 3B). Only small colonies were detected after *cyr1Δ* strain CR216 was incubated one week at room temperature. However, larger colonies began to be detected in about 6% of the restreaked colonies after 2 weeks, and by 4 weeks 100% of the restreaked *cyr1Δ* CR216 cells showed at least one larger when restreaked onto fresh YPD medium. The larger colonies continued to grow faster than the parental *cyr1Δ* strain when restreaked onto fresh agar medium (Fig. 3C).

The faster growing *cyr1Δ* cells were then tested for ability to be induced with GlcNAc to form hyphae. Interestingly, the cells were readily induced by GlcNAc to form filamentous cells, even at an early 90 m time point (Figure 3D). The spontaneous emergence of faster growing cells was not limited to the previously constructed CR216 *cyr1Δ*, as faster growing cells were isolated from newly made *cyr1Δ* strains in the BWP17 strain background by incubating colonies for a period of 3 weeks (Fig. 3C). The pseudorevertants that arose from the *cyr1Δ* mutant in the BWP17 strain background were also induced by GlcNAc to undergo filamentous growth (Fig. 3D). Thus, these results indicate that adenylyl cyclase is not needed to induce hyphal growth.

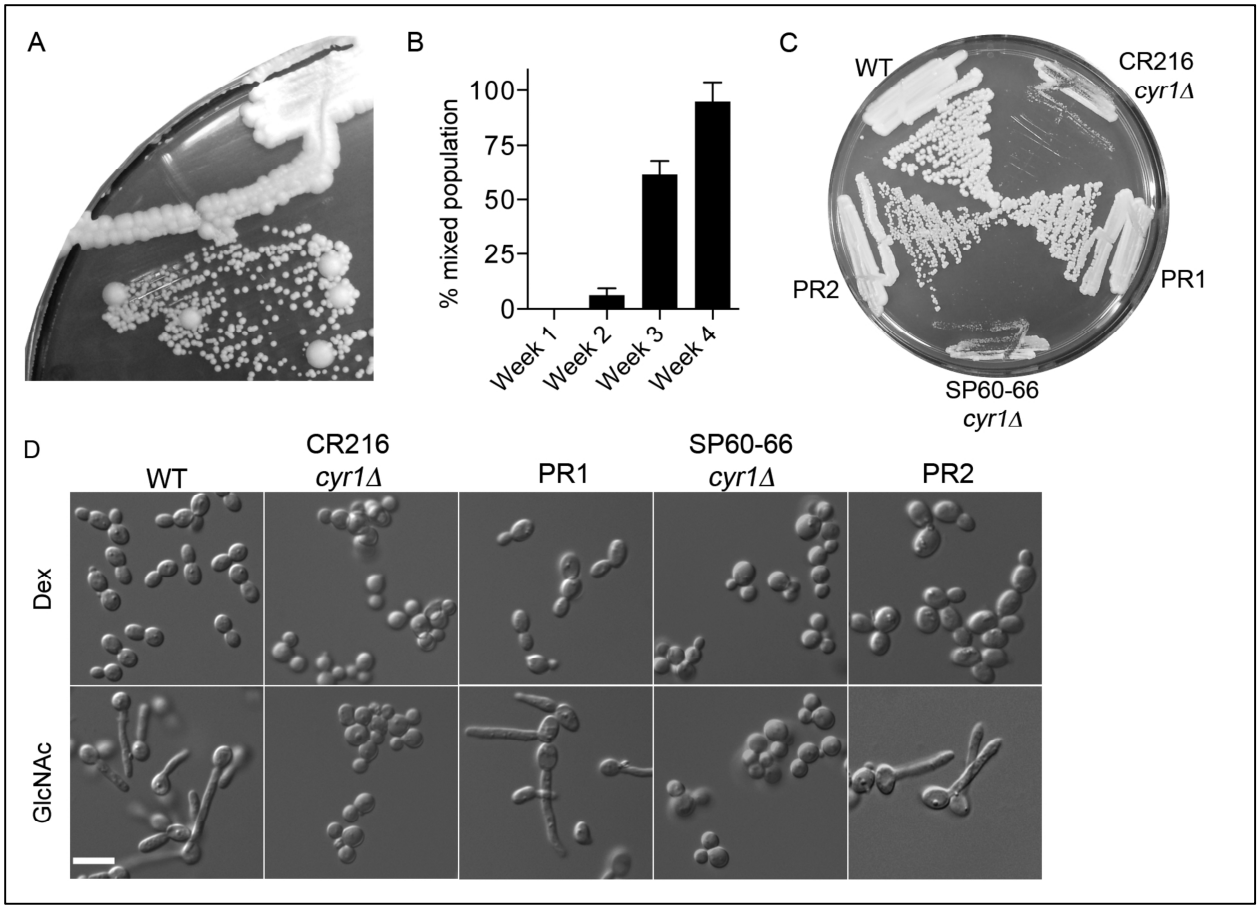

Figure 3. Faster growing pseudorevertants spontaneously arise from *cyr1Δ* cultures over time.

A. Cells streaked onto YPD agar plate that were derived from a representative colony of *cyr1Δ* (CR216) cells that had been incubated at room temperature for 3 weeks. At least two populations of cells are evident: small colonies similar to the *cyr1Δ* parental cells and large colonies of pseudorevertants.

B. Percent of *cyr1Δ* (CR216) colonies incubated for the indicated time that gave rise to mixed populations of large and small cells upon restreaking onto a fresh agar medium plate. Colonies that, when restreaked, gave at least 1 large colony were scored as positive. Results represent the average for 30 colonies assayed this way in each of 3 independent experiments. Error bars indicate SD.

C. Growth rate comparison between the parental *cyr1Δ* cells and pseudorevertants derived from them. Indicated strains were streaked on YPD and grown for 3 days at 30°C. Both the previously reported published *cyr1Δ* strain CR216 and a newly made *cyr1Δ* strain we constructed (SP60-66) gave rise to pseudorevertants that grew at approximately similar rates. Pseudorevertant PR1 was derived from CR216 while PR2 was derived from SP60-66.

D. GlcNAc induction of hyphal growth. Cells grown in SC-URA plus 100 mM dextrose medium, were washed and then resuspended in the same medium containing either 100 mM dextrose or 100 mM GlcNAc and cultured at 37°C for 90 min. As expected, wild type cells formed hyphae but the *cyr1Δ* strains were completely defective. Interestingly, the faster growing pseudorevertants derived from both *cyr1Δ* strains were also stimulated to undergo filamentous growth in GlcNAc medium. Bars, 10 µm.

To ensure that the faster growing cells were not due to contamination, PCR analysis was carried out with 4 sets of primers designed to detect the catalytic domain of *CYR1,* all of which gave negative results (Fig. S1). As an independent way to confirm this, cell extracts were assayed for the presence of cAMP. Cells were cultured in rich media, frozen cell pellets were extracted, and then the levels of cAMP were assessed using an immunoassay kit (Fig. 4A). Wild type control strain DIC185 consistently showed detectable levels of cAMP that agreed well with previous studies (~1.8 pmol mg^{-1} dry weight) (Xu et al., 2008). In contrast, neither the *cyr1Δ* nor the pseudorevertants gave a signal above the limit of detection (Fig. 4A). Similar results were observed for both of the two independent *cyr1Δ* strains and pseudorevertants derived from them. The cAMP assays were also performed on cells that were treated under conditions similar to those used to induce hyphal growth of the pseudorevertants. In brief, cells grown in synthetic medium were induced by addition of bovine calf serum for 30 min, a time previously reported to coincide with the spike in cAMP levels (Maidan et al., 2005). The *cyr1Δ* and their derived pseudorevertants did not show cAMP above the limit of detection for the assay (Fig. 4B). Wild type cells consistently gave a detectable signal, although it was slightly lower in these cells grown in synthetic medium than for cells grown in rich YPD medium in Fig. 4A. These results confirm that the faster growing cells are pseudorevertants and that hyphal induction was stimulated in these cells in the absence of cAMP.

In preliminary studies we also observed fast-growing isolates of *ras1Δ* cells (not shown). These results also demonstrate that care should be taken when working with *cyr1Δ* and *ras1Δ* cells to avoid enrichment for the faster growing pseudorevertants.

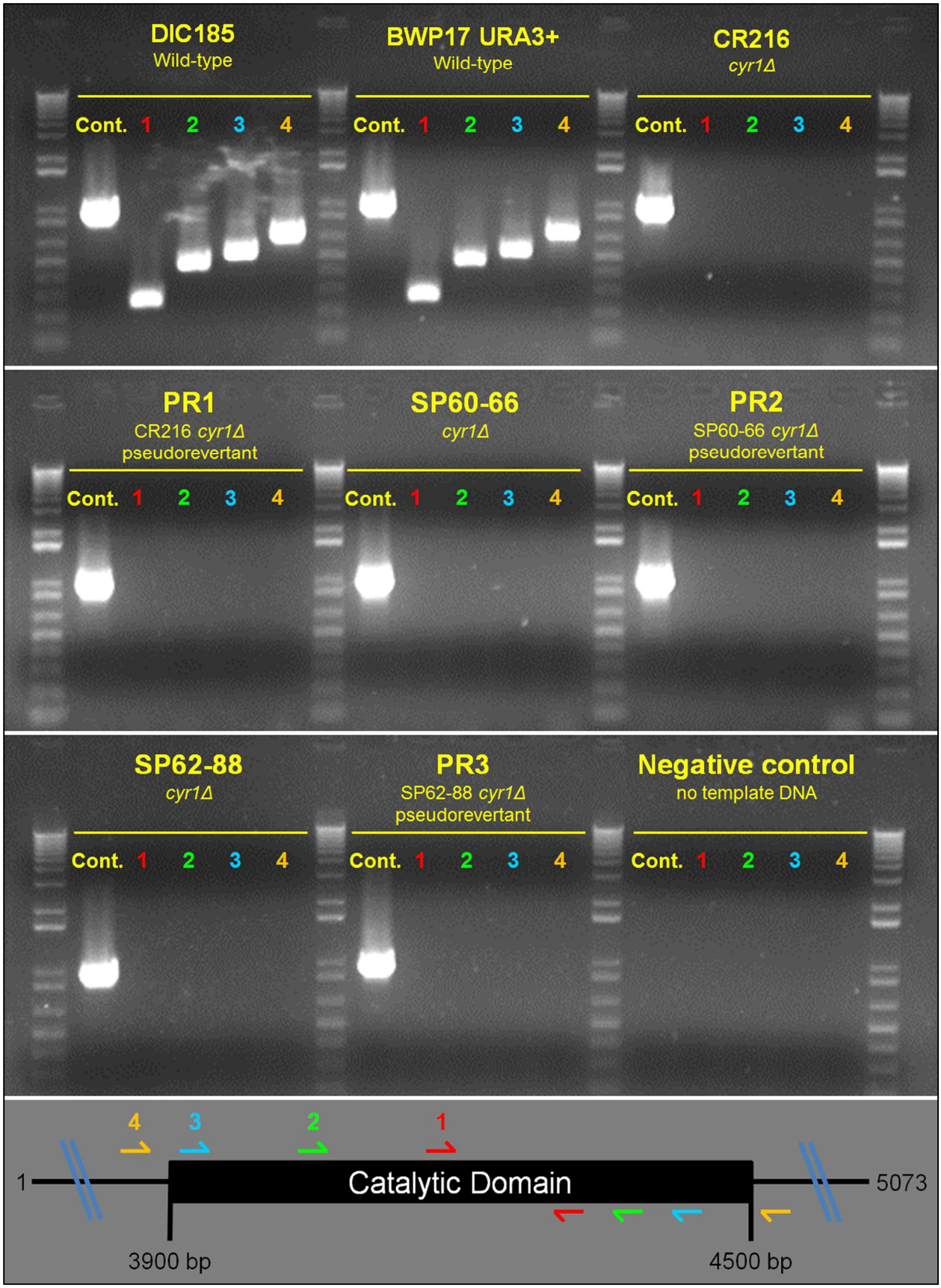
DIC185
Wild-type
Cont. 1 2 3 4
BWP17 URA3+
Wild-type
Cont. 1 2 3 4
CR216
cyr1Δ
Cont. 1 2 3 4
PR1
CR216 cyr1Δ
pseudorevertant
Cont. 1 2 3 4
SP60-66
cyr1Δ
Cont. 1 2 3 4
PR2
SP60-66 cyr1Δ
pseudorevertant
Cont. 1 2 3 4
SP62-88
cyr1Δ
Cont. 1 2 3 4
PR3
SP62-88 cyr1Δ
pseudorevertant
Cont. 1 2 3 4
Negative control
no template DNA
Cont. 1 2 3 4
4
3
2
1
1
Catalytic Domain
5073
3900 bp
4500 bp

Figure S1: Pseudorevertants derived from *cyr1Δ* strains lack the *CYR1* catalytic domain.

The adenylyl cyclase gene *CYR1* contains a 5073 bp open reading frame, in which the catalytic domain that is essential for cAMP production occurs between 3900 and 4500 bp (bottom panel). To ensure that no remnants of the catalytic domain remained in the genomes of the *cyr1Δ* strains and their derived pseudorevertants, PCR analysis was carried out with different sets of primers homologous to the catalytic domain. The PCR products were then separated by electrophoresis on an agarose gel and detected by staining with ethidium bromide. All strains gave a positive PCR result for the control primers that amplified a section of the *BCY1* gene (lanes labeled Cont.). The wild type control strains (DIC185 and BWP17 URA3+) also gave the expected PCR products for four different sets of primers that span the catalytic domain of *CYR1*. In contrast, the *cyr1Δ* strains (CR216, SP60-66, SP62-88) and the pseudorevertants derived from them (PR1, PR2, and PR3) failed to give a PCR product, indicating the absence of the catalytic domain as expected. Note that the lanes of the gels are color coded with numbers that correspond to the indicated primer sets shown on the map at the bottom. As a negative control, a set of reactions was performed in the absence of template DNA ("No Template"). The sets of PCR products are separated by lanes containing molecular weight markers (Invitrogen 1KB+ ladder). The positive control product was detected just below the 1000 base pair band as expected.

The following primers were used in this analysis:

Positive Control Primers (primes to *BCY1* open reading frame), expected product is 980 bp.

BCY1-SP0028F: CCACATCTGAATCACGACGA

BCY1-SP0028R: TCCAATGCTTCCACAGTAGC

Primers for *CYR1* Catalytic domain (in the order they appear in the figure). Expected products are 266, 459, 530, and 690 base pairs respectively.

CYR1-set 1	SP0043F: GACAATTGCGAATTACTGGTGG	
	SP0043R: GCCGGTTATAACATCTGGTTC	
CYR1 set 2	SP0076F: GTGGAGACTCTAATCTCCGT	
	SP0076R: CTCTATTGcAMPATTGGCCCA	
CYR1 set 3	SP0077F: CGTCGAGATAAGCAACAAGT	
	SP0077R: CTGACAGCAATTTGTCCcAMP	
CYR1 set 4	SP0078F: ATAGGCAGAAACAAGCTGCC	
	SP0078R: TTCCGGCATTTTCATTGCCC	

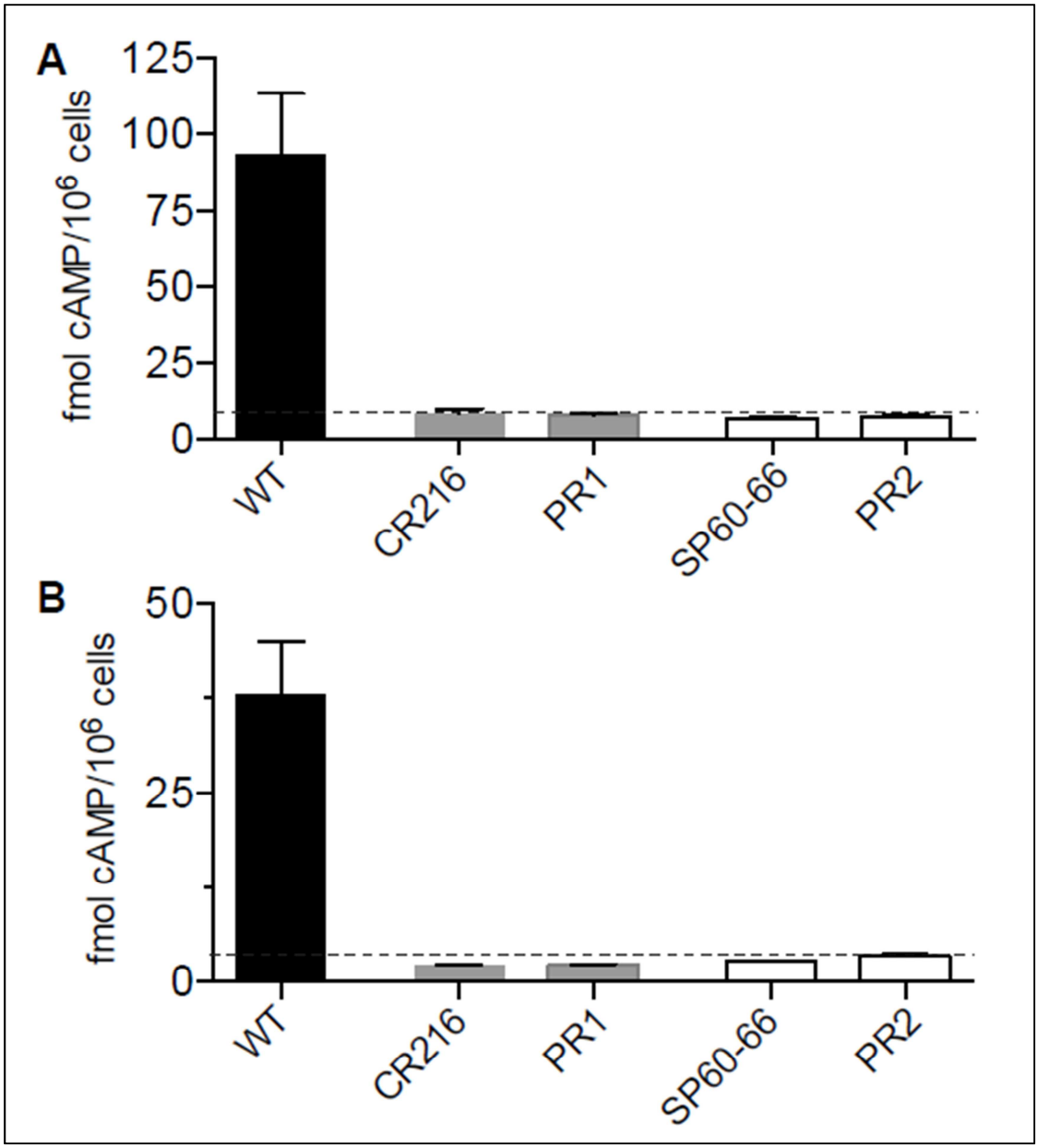

Figure 4: Pseudorevertants do not contain detectable cAMP.

A. Wild type, *cyr1Δ*, and derived pseudorevertant cells were grown overnight in rich YPD medium, cAMP was extracted, and then cAMP levels were assayed using a GE Amersham cAMP EIA-Immunoassay kit with the non-acetylation protocol. The pseudorevertant strains PR1 and PR2 showed low signals that were at or below the limit of detection, indicating that no cAMP could be detected, similar to their parental strains *cyr1Δ* strain CR216 and *cyr1Δ* strain SP60-66.

B. The strains described above were grown in synthetic SC-URA medium with 100 mM dextrose and the induced with 30% serum at 37˚ C for 30 min. cAMP was extracted as in panel A and assayed using the more sensitive acetylation protocol to detect cAMP. The *cyr1Δ* strains and their derived pseudorevertants contained no detectable cAMP under these conditions, similar to the results obtained with rich medium in panel A. The results for each strain represent the average cAMP value obtained from the analysis of at least four independent colonies. The dashed line indicates the limit of detection of the assay. Error bars indicate SD.

3.34 Pseudorevertant strains form true hyphae

The ability of the pseudorevertants to form elongated filamentous cells was examined further to determine if they were being induced to form true hyphae. There are many types of filamentous growth, and not all are considered to be true hyphal growth. Hyphal growth is characterized by chains of elongated cells with parallel cell walls, a first septum distal to the bud neck, and multiple nuclei separated by septae (Sudbery *et al.*, 2004). Pseudohyphal cells are characterized as chains of elongated buds with a first septum at the neck of the mother cell and indentations at subsequent septae. In addition, some mutants can form elongated cells that resemble hyphal filaments, such as elongated buds formed by septin mutants or in response to oxidative stress (Li *et al.*, 2012, Gauthier & Klein, 2008). To determine if *cyr1Δ* pseudorevertants form true hyphae, two independent pseudorevertant strains were induced with GlcNAc. Microscopic analysis at relatively low magnification revealed that germ tubes could extend long hyphal cells for both the wild type and the pseudorevertants (Figure 5A). To determine if these cells formed true hyphae, they were stained with Pontamine Fast Scarlet 4B to detect cell wall chitin and with Hoechst 33342 to detect nuclear DNA. As expected for true hyphae, wild type cells formed the first septum distal to the bud neck and subsequent nuclei were separated by septae (Figure 5B). Similar results were observed for two independently derived pseudorevertant strains. A higher magnification image of the septae indicated that the pseudorevertants were similar to the wild type in forming a flat zone of cell wall across the septum. These results indicate that the pseudorevertants are capable of forming true hyphae.

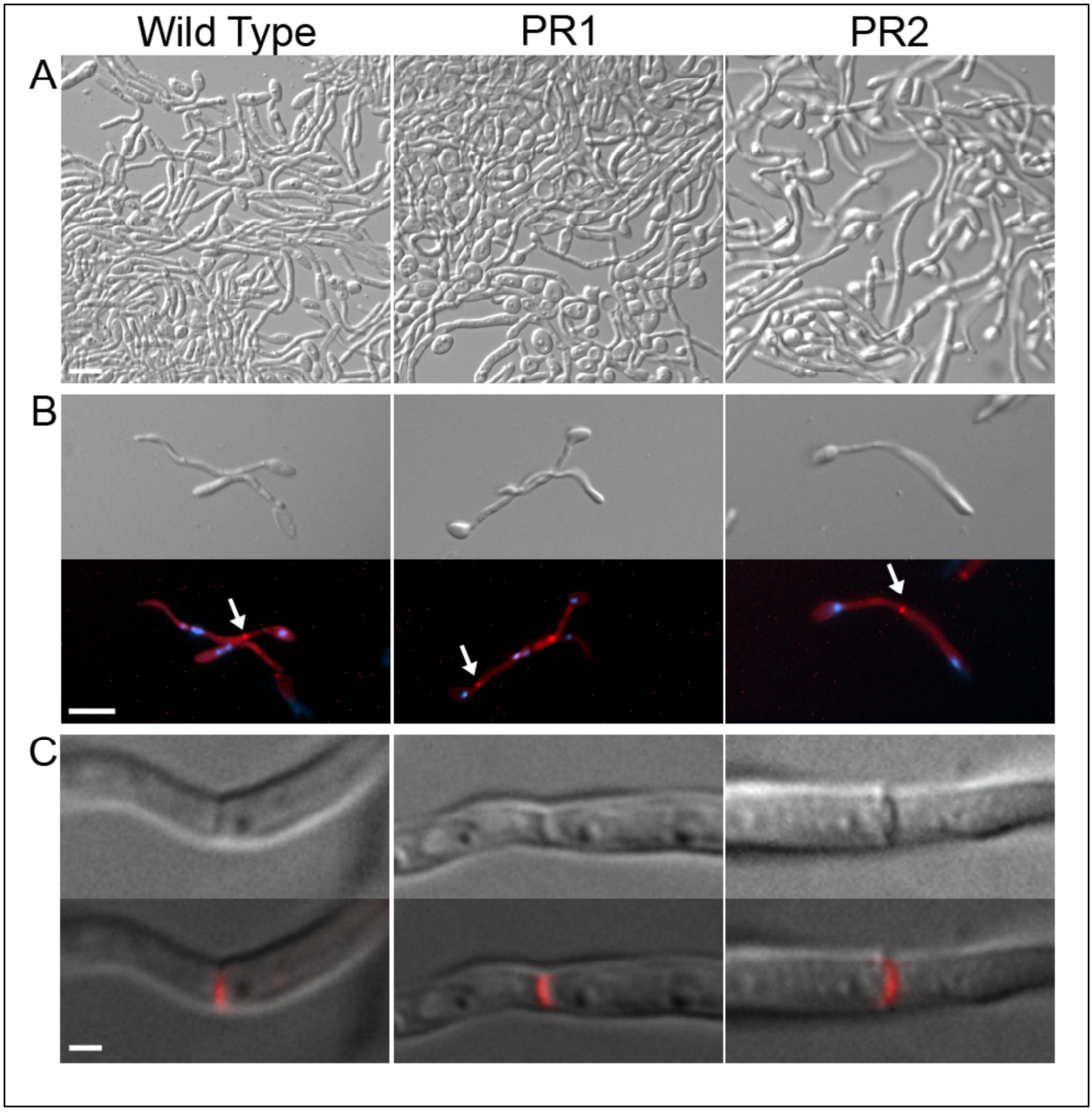

Figure 5: Pseudorevertants undergo true hyphal growth

A. Wild type cells (DIC185), and pseudorevertant cells derived from both *cyr1Δ* strains (CR216 and SP60-66) were cultured overnight in 100 mM GlcNAc at 37˚C and then photographed at low magnification. Bar, 30 µm.

B. Hyphal cells from the cultures in panel A were stained with Hoechst 33342 to detect DNA (Blue) and Pontamine Fast Scarlet 34 (Red) to detect cell wall chitin. Hyphal cells from all

three strains displayed characteristics of true hyphae including a first septum distal to the mother cell (arrow). Bar, 10 µm.

C. Upper panels show a close-up view of a single septum. Lower panels show the septum image merged with Red staining from Pontamine Fast Scarlet 34 to verify the presence of the septum. Bar, 1 µm.

3.35 Pseudorevertants induce hyphal morphology and gene expression in response to some but not all inducers

Activation of the cAMP pathway has been reported to play an important role in the induction of a specific set of genes in hyphae. It is thought that the hyphal repressor Nrg1 must be evicted from the promoters of hyphal-specific genes by a process that is activated by an increase in signaling through the cAMP pathway and accordingly, a *cyr1Δ* mutant is defective at this (Lu et al., 2011). To determine if hyphal genes are induced during hyphal growth in pseudorevertant strains, cells were engineered to express a reporter gene consisting of GFP under the control of the hyphal-specific promoter of *HWP1*. It is important to note that the *cyr1Δ HWP1-GFP* strain was constructed by first introducing the *HWP1-GFP* construct into a heterozygous strain containing one copy of *CYR1*, which was then deleted as the last step in the procedure. We had found that when *cyr1Δ* strains were put through the standard lithium acetate transformation procedure the resulting transformants were frequently pseudorevertants. In contrast, deleting the remaining copy of *CYR1* as the last step allowed isolation of the expected slower growing cells rather than pseudorevertants. The slow growing *cyr1Δ HWP1-GFP* strain was then used to isolate a faster growing pseudorevertant by incubating for 3 weeks on solid media as described earlier.

Strains carrying the *HWP1-GFP* reporter gene were treated with GlcNAc, serum, high pH, or CO_2 to determine how they would respond to these different hyphal inducers (Fig. 6). As expected, wild type cells formed hyphae and expressed GFP under all inducing conditions, whereas the *cyr1Δ HWP1-GFP* strain neither formed filamentous cells nor showed *GFP* expression. The pseudorevertant *cyr1Δ HWP1-GFP* strain formed hyphal cells and expressed *GFP* when induced with GlcNAc, serum, and high pH, providing additional evidence that they are induced to undergo a typical hyphal induction.

Interestingly, growing cells in an environment enriched in CO_2 did not induce hyphae or the *HWP1-GFP* reporter gene in the pseudorevertant strain. This is consistent with previous reports that adenylyl cyclase is a sensor for CO_2 in *C. albicans* and other organisms (Hall et al., 2010). After entering cells, CO_2 is converted to bicarbonate ion, which is then thought to bind the catalytic lysine of adenylyl cyclase, increasing the specific activity of the cyclase and thereby increasing cAMP levels. These data therefore support the model that bicarbonate simulates hyphal growth by activating adenylyl cyclase.

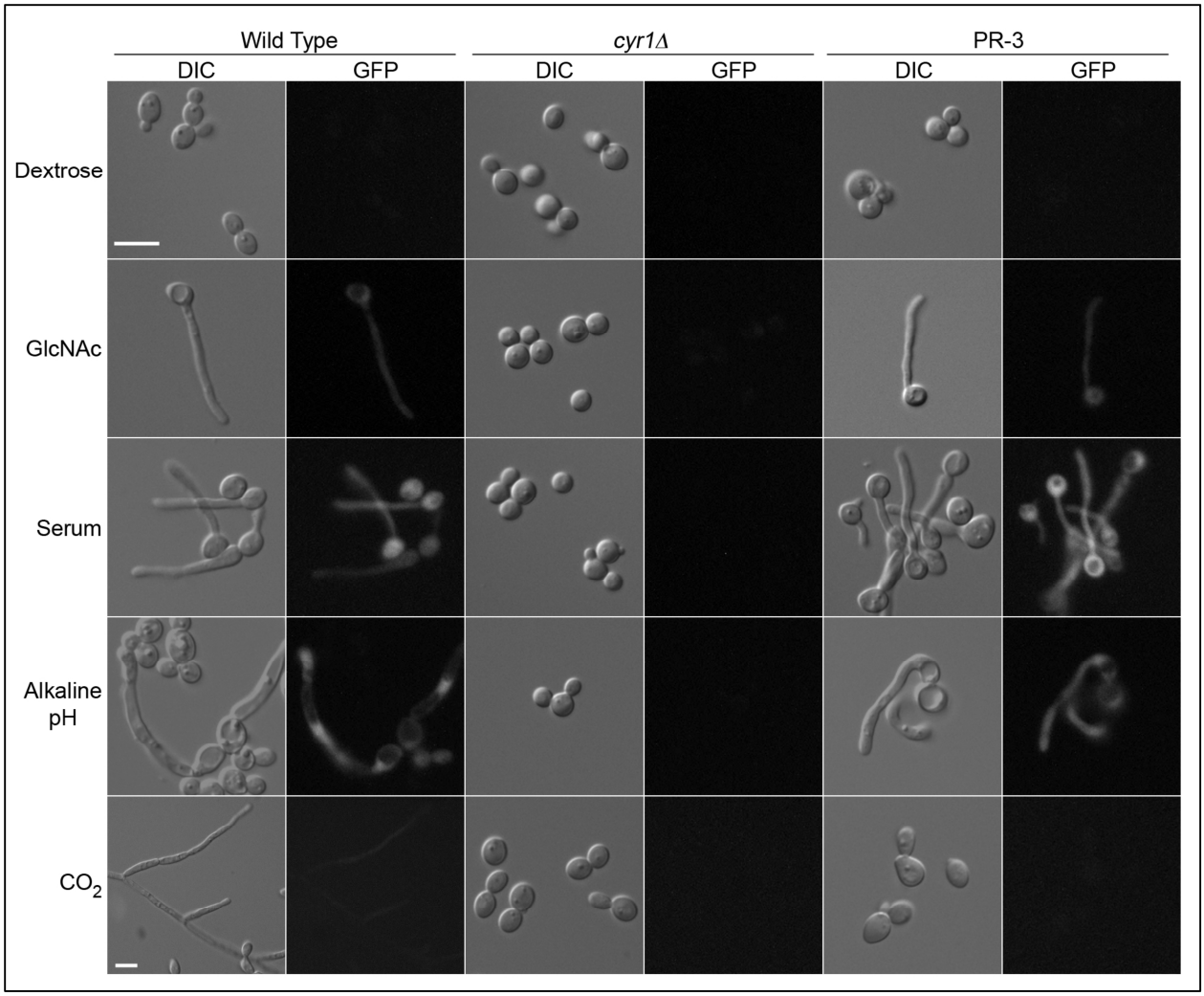

Figure 6. Pseudorevertants induce hyphal morphology and gene expression in response to many inducers, but not CO_2.

Strains carrying the *HWP1-GFP* reporter gene were incubated under the indicated hyphal inducing conditions and then photographed using light microscopy to detect cell morphology and fluorescence microscopy to determine if GFP was produced. As expected, all of the inducers stimulated the wild type control strain to form hyphae and produce GFP, whereas the *cyr1Δ* strain showed no detectable hyphae or GFP. In contrast, the pseudorevertant strain PR3 showed induction of filamentous cells and GFP levels similar to the wild type under all conditions except for CO_2. All inductions were performed at 37°C. GlcNAc induction was performed using SC-URA medium with 100 mM GlcNAc and was visualized at 90 min. Serum induction was

performed in SC-URA plus 100 mM dextrose and 30% bovine calf serum and was visualized after 90 min. pH induction of hyphal growth was performed by culturing cells overnight in SC medium buffered to pH 8 with 150 mM HEPES. CO_2 induction was performed by culturing cells overnight in a chamber with 5% CO_2.

3.36 Pseudorevertants show some similarity to opaque cells, but are not dependent on WOR1

As described above, after 4 weeks incubation on solid agar medium essentially 100% of *cyr1Δ* colonies restreaked onto a fresh agar plate will give rise to a subset of cells that grow faster. We also observed that the relative proportion of large colonies increased with time. When 4 week old colonies were restreaked onto fresh medium, the large pseudorevertant colonies accounted for about 3% of total colonies that grew up (Fig. 7A). This relatively high spontaneous rate of appearance of the pseudorevertants raised the possibility that the suppression of the slow growth phenotype may be due to an epigenetic change, rather than a mutation in the genome. The most commonly studied spontaneous epigenetic change in *C. albicans* is the White-Opaque switch (Soll, 2009). Opaque cells are named for their distinctive colony morphology and have also been shown to have a very distinct pattern of gene expression, including metabolic genes, compared to the White phase cells (Mehrabi *et al.*, 2009). To determine if the pseudorevertants show similarity to Opaque cells, they were grown on medium containing Phloxine B; a dye that stains Opaque cells dark magenta. Interestingly, the large pseudorevertant colonies stained deep magenta and appeared somewhat flatter, as expected for Opaque cells. In contrast the smaller colonies stained were lighter colored, as expected for White phase cells (Fig. 7B).

To examine this further, the *WOR1* gene was deleted in a pseudorevertant strain. *WOR1* encodes a transcription factor that is considered to be the master regulator of the transition from White to Opaque phase (Zordan et al., 2007). However, deletion of *WOR1* in a pseudorevertant strain had no effect on the strain's Phloxine B staining, growth rate, or ability to induce hyphae (Figure 7C, D, E). In addition, out of a total of 35 pseudorevertant strains tested by PCR, 28 tested positive for the presence of both *MTLa1* and *MTLα1*, including strains PR1, PR2, and PR3

that were used in this study. In the strain background used in these studies, *MTLa1* or *MTLα1* must be lost before a strain becomes capable of the White-Opaque switch (Miller & Johnson, 2002). Furthermore, although Opaque cells will switch back to the White phase at 37˚ C, incubating *cyr1Δ* colonies at 37˚ C did not prevent the appearance of pseudorevertants, and growth of cells at 37˚C did not alter their ability to stain more darkly with Phloxine B. These results suggest that in spite of some similarities with Opaque cells, the *cyr1Δ* pseudorevertants arise through a distinct mechanism.

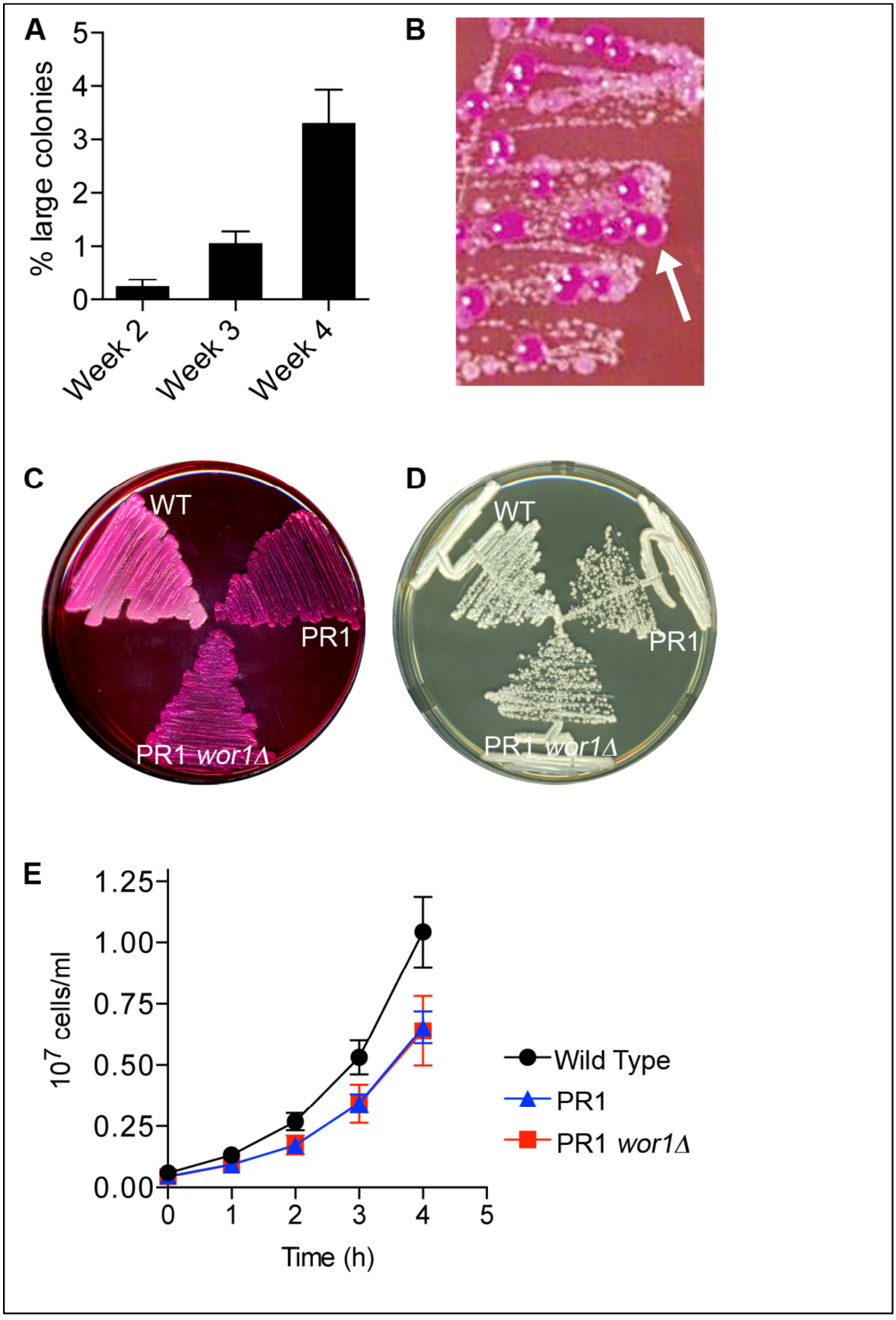
A
% large colonies
0
1
2
3
4
5
Week 2
Week 3
Week 4
B
C
WT
PR1
PR1 wor1Δ
D
WT
PR1
PR1 wor1Δ
E
10^7 cells/ml
0.00
0.25
0.50
0.75
1.00
1.25
0
1
2
3
4
5
Time (h)
Wild Type
PR1
PR1 wor1Δ

Figure 7. Pseudorevertants show increased Phloxine B staining, but not other characteristics of Opaque cells.

A. The *cyr1Δ* strain CR216 was incubated at room temperature on YPD agar medium for the indicated time, restreaked onto fresh medium, and then the resulting colonies were assessed to determine the percent of faster-growing large colonies rather than the expected small colonies. The results represent the average for 6 colonies aged for two weeks, 59 colonies aged for three weeks, and 91 colonies aged for four weeks.

B. The *cyr1Δ* cells (CR216) were incubated 3 weeks on YPD agar plates and then streaked onto a YPD plate containing 45 µg ml^{-1} Phloxine B. Larger pseudorevertant colonies were observed to stain magenta (arrow), similar to that expected for cells in the Opaque phase.

C. Wild type (DIC185), a *cyr1Δ* pseudorevertant (PR1) derived from strain CR216, and its *wor1Δ* derivative (PR1-*wor1Δ*) were streaked onto YPD agar plate containing 45 µg ml^{-1} Phloxine B, showing that deletion of *WOR1* did not change Phloxine B staining of the pseudorevertant.

D. The strains described in panel C were streaked for single colonies on YPD and incubated at 30˚ C for 2 days.

E. The strains described in panel C were grown in liquid YPD media at 30˚ C. Panels D and E show that deletion of *WOR1* does not affect the growth rate of the pseudorevertant strain PR1.

3.4 Discussion

Activation of adenylyl cyclase is sufficient to induce hyphal growth in *Candida albicans*, since hyperactive adenylyl cyclase alleles or addition of exogenous cAMP promote filamentous morphogenesis (Sabie & Gadd, 1992, Bai et al., 2011). Adenylyl cyclase and cAMP signaling has also been inferred to be necessary for the ability to respond to most inducers, because *cyr1Δ* mutants are defective in forming hyphae in response to a wide range of hyphal inducing conditions (Wang, 2013, Whistler & Rine, 1997). However, an important caveat is that cAMP is not exclusively involved in hyphal signaling; it is also important for normal cellular function. The *cyr1Δ* cells show reduced expression of many metabolic genes, including those encoding ribosomal proteins, RNA polymerase subunits, and enzymes involved in the Krebs cycle, pyrimidine metabolism, and the synthesis of heme and sterols (Harcus et al., 2004). Consistent with this, *cyr1Δ* cells have abnormal shape, size, and a three-fold growth rate defect (Rocha et al., 2001, Doedt et al., 2004). This raised the question as to whether the hyphal inducers must act by stimulating adenylyl cyclase, or if a normal basal level of cAMP is required for cells to be competent to undergo highly polarized hyphal morphogenesis.

3.41 Basal cAMP level is important for hyphal induction

To examine the role of the basal level of cAMP in hyphal signaling, cells exposed to different levels of the cell permeable compound db-cAMP were examined for ability to undergo filamentous growth in response GlcNAc. Interestingly, GlcNAc was able to induce hyphal growth at low levels of db-cAMP that were not sufficient to induce hyphae on their own (Fig. 2). Similar results were reported in a recent study, which showed that db-cAMP supplementation rescued the ability of a *ras1Δ* mutant to be induced with GlcNAc to form hyphae (Davis-Hanna et al., 2008). These results indicate that the basal level of cAMP is critical for induction by

GlcNAc, rather than a spike in adenylyl cyclase activity. This suggests that the lower basal levels of cAMP found in *ras1Δ* and *csc25Δ* mutants (Maidan et al., 2005) may contribute to their hyphal defects, rather than the inability to induce adenylyl cyclase.

3.42 Pseudorevertants form hyphae in the absence of adenylyl cyclase.

In the course of these studies we discovered that *cyr1Δ* strains give rise to pseudorevertants that grow nearly as well as the wild type control strain (Fig. 3). These faster-growing cells were confirmed to be pseudorevertants because they lacked the adenylyl cyclase gene (Sup. Fig. 1) and also lacked detectable cAMP (Fig. 4). They arise at a high frequency, suggesting they may occur by an epigenetic mechanism, although they were independent of the Wor1 transcription factor that is known to regulate a type of epigenetic change in *C. albicans* known as White-Opaque switching (Fig. 7). Interestingly, the pseudorevertants could be stimulated to form true hyphae (Fig. 5), and not abnormal filamentous growth that has been reported for septin mutants or cells exposed to oxidative stress (Li et al., 2012, Gauthier & Klein, 2008). The pseudorevertants were also similar to the wild type in that they could induce a hyphal-specific reporter gene, *HWP1-GFP* (Fig. 6). These results provide strong evidence that adenylyl cyclase is not essential for hyphal induction.

The ability of GlcNAc, serum, and alkaline pH to stimulate hyphae in a cAMP-independent manner highlights the importance of other signaling pathways in regulating filamentous growth (Fig. 6). GlcNAc is known to activate a cAMP-independent pathway, since the genes needed for GlcNAc catabolism can be induced in a *cyr1Δ* mutant (Naseem *et al.*, 2011). Other studies have implicated Rac1 and the Cek1 MAP kinase in signaling hyphal growth (Hope

et al., 2010), and there is also a contact-sensing pathway that involves the membrane protein Dfi1 (Mbonyi *et al.*, 1988). In addition, these results may help explain the relationship between the cAMP pathway and induction of hyphal growth by alkaline pH, which is mediated by the Rim101 pathway in a manner that does not obviously intersect with adenylyl cyclase (Davis, 2009). Alternatively, some inducers may activate the PKA pathway downstream of cAMP. The existence of additional pathways for hyphal induction is also consistent with the differential ability of various hyphal inducing conditions to also stimulate hyphal gene expression, rather than inducing the same relative levels of gene expression as would be expected if there was only a single main pathway (Martin *et al.*, 2013, Naseem et al., 2015). The pseudorevertants will therefore provide a new tool to study these alternate hyphal induction pathways without interference from the effects of cAMP levels on growth.

Although the pseudorevertants were induced by several different stimuli to form hyphae, they did not induce hyphae in response to CO_2. This is consistent with previous studies showing that CO_2 becomes converted into bicarbonate in cells, which then acts directly on adenylyl cyclase to stimulate cAMP production (Hall et al., 2010). In addition, although some inducers can stimulate cAMP-independent pathways, they may also activate adenylyl cyclase. For example, serum may be such a potent inducer of hyphal morphogenesis because it can induce both adenylyl cyclase and cAMP-independent pathways. There is likely to be synergy between these pathways as stimulation of adenylyl cyclase can induce the level of other signaling components (Slutsky *et al.*, 1987).

3.43 Adenylyl cyclase is important in diverse fungal pathogens

The results of this study have important implications for the study of other fungal pathogens, as the cAMP pathway has been reported to regulate virulence functions in a diverse set of pathogenic fungi. For example, cAMP levels govern the morphological change from filamentous growth to the pathogenic yeast form in the dimorphic fungus, *Paracoccidioides brasiliensis* (Jiang *et al.*, 1998), virulence functions in the mold *Aspergillus fumigatus* (Munder & Küntzel, 1989), and in the budding yeast *Cryptococcus neoformans*, the cAMP pathway controls the formation of the polysaccharide capsule and deposition of melanin into the cell wall, both of which are key virulence functions (Van Aelst *et al.*, 1990). Since the cAMP pathway also regulates growth properties in other fungi as it does in *C. albicans*, it will be similarly important to distinguish between a direct role of adenylyl cyclase in regulating virulence functions and an indirect effect due to more global effects on metabolism.

Table 1. *C. albicans* strains used in this study.

Strain	Parent	Genotype
BWP17	SC5314	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4*
DIC185	BWP17	*ura3Δ::λimm434/URA3 his1:hisG/HIS1 arg4::hisG/ARG4*
		ura3Δ::λimm434/URA3 his1::hisG/his1::hisG
BWP17URA3+	BWP17	*arg4::hisG/arg4::hisG*
	BWP17	*csc25Δ::SAT1-FLIP/csc25Δ::ARG4 ura3Δ::λimm434/*
SP53-1	URA3+	*URA3 his1::hisG/HIS1 arg4::hisG/arg4*
	BWP17	*ras1Δ::SAT1-FLIP/ras1Δ::ARG4 ura3Δ::λimm434/ URA3*
SP54-1	URA3+	*his1::hisG/HIS1 arg4::hisG/arg4*
	BWP17	*cyr1Δ::SAT1-FLIP/cyr1Δ::ARG4 ura3Δ::λimm434/ URA3*
SP60-66	URA3+	*his1::hisG/HIS1 arg4::hisG/arg4*
		cyr1Δ::SAT1-FLIP/cyr1Δ::ARG4 ura3Δ::λimm434/ URA3
PR2	SP60-66	*his1::hisG/HIS1 arg4::hisG/arg4*
	BWP17	*HWP1-GFP-HIS1 ura3Δ::λimm434/URA3*
SP57-1	URA3+	*his1::hisG/his1::hisG arg4::hisG/ARG4*
		HWP1-GFP-HIS1 cyr1Δ::SAT1-FLIP/cyr1Δ::ARG4
	BWP17	*ura3Δ::λimm434/URA3 his1::hisG/his1::hisG*
SP62-88	URA3+	*arg4::hisG/arg4::hisG*
		HWP1-GFP-HIS1 cyr1Δ::SAT1-FLIP/cyr1Δ::ARG4
		ura3Δ::λimm434/URA3 his1::hisG/his1::hisG
PR3	SP62-88	*arg4::hisG/arg4::hisG*
		ura3::λimm434/ura3::1imm434 cdc35Δ::hisG-URA3-
CR216	CAI4	*hisG/cdc35Δ::hisG*
		ura3::λimm434/ura3::λimm434 cdc35Δ::hisG-URA3-
PR1	CR216	*hisG/cdc35Δ::hisG*
		wor1Δ::FRT/wor1Δ::SAT1-FLIP
		ura3::λimm434/ura3::λimm434 cdc35Δ::hisG-URA3-
PR1-*wor1Δ*	PR1	*hisG/cdc35Δ::hisG*

Chapter 4: Future Directions

4.1 Identification of the suppressor mutations in *cyr1Δ* pseudorevertants

The *cyr1Δ* pseudorevertants are an interesting tool for probing the molecular mechanisms of hyphal induction in *C. albicans* in the absence of signaling through adenylyl cyclase and cAMP. It would be prudent therefore to identify the means of suppression. Suppression of cAMP pathway deletion phenotypes is better studied in *S. cerevisiae,* where it was found that an increase in downstream or parallel signaling by gene overexpression can rescue the phenotype of cAMP pathway deletion mutants.

Deletion of the *CSC25, RAS1,* or *CYR1* orthologues is lethal in *S. cerevisiae.* However, stable deletion mutants of the *CSC25* and *RAS1* orthologues can be obtained if the strain is engineered to overexpress the cAMP-dependent Protein Kinase (PKA) catalytic domain, *TPK1,* which is downstream of cAMP. (Whistler & Rine, 1997) Overexpression of *SCH9* (Suppressor of Cdc25, High Copy), a kinase similar to but distinct from PKA, can suppress deletion of *S. cerevisiae CSC25, RAS1,* and *CYR1* orthologues, as well as deletion of all 3 PKA catalytic subunits (Toda et al., 1988). The *Schizosaccharomyces pombe* orthologue of *SCH9, sck1,* was able to suppress the deletion of the cAMP pathway orthologue in that organism as well (Jin et al., 1995).

Thus it is likely that overexpression of *SCH9,* PKA catalytic subunits, or another protein kinase could be responsible for suppression in *cyr1Δ* pseudorevertants. Theoretically, activating mutations could achieve the same effect but due to the relatively high rate of emergence of pseudorevertants, nearly 4% of cells in aged colonies demonstrate a suppressed phenotype as detailed in Chapter 3 Figure 7A, we suspect overexpression. This is because overexpression can be caused by copy number variation, gene regulation, or both. Copy number variation through

aneuploidy and gene duplication are well documented in *C. albicans*. Its tolerance of ploidies from 1 to 4N underscores its genomic plasticity, and allows it to adapt to a wide range of adverse conditions (Selmecki *et al.*, 2010). An example of this is that propagation on media with Sorbose as the main carbon sources predictably leads to strains losing one homolog of Chromosome 5, which confers a growth rate advantage on this sugar (Kabir *et al.*, 2005). Gene duplication is also possible. One group studying an unrelated phenomenon noticed that *URA3*, which encodes an enzyme in the uracil synthesis pathway, was frequently present in multiple copies in their strains, even reaching 18 copies in one strain (Wartenberg *et al.*, 2014).

Additionally, the White-Opaque epigenetic switch is known to reconfigure metabolism; a switch to the Opaque phase was observed to lead to upregulation of 221 ORFs, 20% of which were involved in metabolism and 8% involved in cellular energy production (Lan et al., 2002). It is possible that a White-Opaque switch has restored expression of the metabolic genes downregulated by deletion of *CYR1*, as detailed in Chapter 3. Though deletion of *WOR1* was not found to affect *cyr1Δ* pseudorevertants' growth rate or ability to stain on Phloxine B, it should be noted that *WOR1* is one in a family of *WOR* transcription factors that regulate the White-Opaque switch (Lohse *et al.*, 2013, Zordan et al., 2007). Additionally, switching to Opaque-like morphologies was observed in strains deleted for *WOR1* and also strains that had not lost a copy of the fungal Mating-type locus (MTL) *(Tao et al., 2014, Xie et al., 2013).* Thus, that an Opaque or Opaque-like epigenetic switch has occurred is not precluded.

To assay for these changes and others not predicted, next generation DNA sequencing and RNA-seq is being performed on *cyr1Δ* pseudorevertants. Aneuploidies and copy variations can be inferred based on the relative number of reads for genes. Activation of the Opaque-phase

transcription program can be determined by the relative expression of Opaque-specific genes *OP4*, *SAP1*, and *PHO89* (Tao et al., 2014).

4.2 Searching for alternative pathways using *cyr1Δ* pseudorevertants

Ras1 has so far been discussed as an upstream activator of Cyr1 Adenylyl cyclase. The mammalian and *S. cerevisiae* the *RAS1* orthologues are known to regulate the MAP Kinase pathway, which itself is known to promote filamentous growth in both *S. cerevisiae* and *C. albicans (Leberer et al., 2001).* Ras1 signaling through the *C. albicans* MAP Kinase pathway has been suggested but not conclusively demonstrated. As a first step in determining if Ras1 signals through the MAP Kinase or in any other pro-hyphal signaling pathway, the *RAS1G13V* allele could be introduced into *cyr1Δ* pseudorevertants. It is known that the hyperfilamentation caused by the constitutively activated *RAS1G13V* allele can blocked by deletion of *CYR1(Rocha et al., 2001).* As discussed at length in Chapter 3, this may not be indicative of lack of signaling, but may be due to the secondary effects of the deletion of *CYR1*. Expression in the pseudorevertants however would be expected to circumvent this problem and allow intact signaling to be visualized.

Activation of PKA by alternative pathways has been demonstrated in *S. cerevisiae (Peeters et al., 2006, Lu & Hirsch, 2005).* It was demonstrated that Kelch-repeat containing proteins Krh1/2, regulated by the Gα protein Gpa2, directly interact with the PKA complex. Interaction with the complex strengthens the interaction between the PKA catalytic subunits and negative regulatory subunit Bcy1, thereby downregulating PKA activity. Binding of Gpa2 by Krh1/2 causes release of the PKA complex, thus increasing PKA activity. Gpa2 is regulated by the G-protein coupled receptor Gpr1, which responds to sucrose and dextrose in *S. cerevisiae* and

C. albicans (Maidan et al., 2005). Thus, sugar sensing can regulate PKA activity independently of adenylyl cyclase and cAMP.

To determine if cAMP-independent regulation of PKA contributes to the ability of GlcNAc, Serum, and extracellular pH to promote hyphal growth, the phosphorylation of Threonine-206 of Efg1 will be monitored in *cyr1Δ* pseudorevertants induced to switch to hyphal growth using the aforementioned conditions. As mentioned in the Introduction, Efg1 is suspected to be phosphorylated at position 206 by PKA when cells are induced to form hyphae (Bockmuhl & Ernst, 2001). In *S. cerevisiae*, the phosphorylation of Sfl1 and Msn2 was used to assay PKA activity (Lu & Hirsch, 2005). Though *C. albicans* contains *SFL1* and *MSN2* orthologues, less is known about their phosphorylation by PKA than is known about Efg1, making Efg1 the preferred choice for measuring PKA activity in *C. albicans* cells.

4.3 Toward development of novel drugs and therapeutic schemes

As mentioned before, strains deficient in hyphal growth are attenuated for virulence in the mouse model of disseminated candidiasis (Zheng & Wang, 2004). Thus, molecules that prevent hyphal induction have potential as novel therapeutics. Prior to my thesis studies, Cyr1 Adenylyl cyclase of the cAMP-pathway was an attractive therapeutic target. Cyr1 is clearly important for normal cellular function and is especially critical in *C. albicans* as its genome encodes only a single Adenylyl cyclase (Rocha et al., 2001). Though the catalytic subunit is conserved from bacteria to humans, fungal specific inhibitors of AC could be developed.

Initially, inhibitors of Cyr1 would be expected to cause a decreased cellular health, lowered expression of virulence factors, inability to switch to hyphal growth, a growth rate defect, and attenuate virulence, similar to non-pseudorevertant *cyr1Δ*. Our finding that *cyr1Δ* pseudorevertant strains spontaneously arise predicts that resistance to these effects can and

would develop. However, our findings in Chapter 3, Figure 6 predict that though pseudorevertant-like cells treated with a Cyr1 inhibitor would regain their general cellular health and competence to switch to hyphal growth in response to several inducers, they would remain defective in sensing the physiological inducers CO_2/Bicarbonate and MDPs. This is because these inducers act by increasing the specific activity of Adenylyl cyclase, which would be blocked by the inhibitor (Xu et al., 2008, Klengel et al., 2005). Strains deficient in sensing CO_2/Bicarbonate were found to be defective for virulence in the *Drosophila melanogaster* infection model (Klengel et al., 2005). It is therefore reasonable to assume that a switch to a pseudorevertant-like phenotype would not result in full recovery of virulence during treatment with an inhibitor of Cyr1.

C. albicans Cyr1 inhibitors would be expected to initially slow growth severely, inhibit tissue invasion, and promote clearance. Whether or not these inhibitors would continue to provide meaningful anti-candida effects after pseudorevertant-like strains arise may be predicted by assaying the virulence of *cyr1Δ* pseudorevertants. In essence, *cyr1Δ* pseudorevertants approximate a strain well-adapted to an ever-present and perfect Cyr1 inhibitor. In addition to identifying what lasting effects on virulence the inhibition of Cyr1 may impart, synergy with current antifungals could be determined. Though *cyr1Δ* pseudorevertants have suppressed a large part of the blow to cellular health incurred through deletion of *CYR1*, as can be seen in Chapter 3 Figure 7, they have not reconstituted a completely normal growth rate. It is unlikely that they have the same resistance as wild-type to current antifungal drugs, providing opportunities for dual drug therapy.

Conceptually, a single central factor through which all hyphal signaling is integrated would make the ideal drug target for a therapy designed around preventing hyphal growth. The

impetus to identify a single necessary, targetable mechanism for hyphal growth, or virulence in general, has fueled research into *C. albicans* hyphal signaling. My thesis studies, along with others, indicate that no such central mechanism exists; instead hyphal inducers are sensed by multiple pathways, cAMP-dependent and independent, and all contribute to hyphal growth. It is therefore important to determine which sets of hyphal signaling pathways, when blocked, cause the most severe attenuation of hyphal growth. Targeting Cyr1 of the cAMP-pathway along with a cAMP-independent hyphal signaling pathway may circumvent the problem posed by pseudorevertant-like strains arising during therapy with a Cyr1 inhibitor.

The finding that both cAMP-dependent and cAMP-independent pathways are fully capable of inducing hyphal growth and HSGs is critical for development of therapeutics designed to block hyphal growth. In addition, it contributes to our understanding of the fascinating biological sensing-and-response system that controls hyphal growth in *Candida albicans*. Lastly, a new tool in the form of the *cyr1Δ* pseudorevertants has been identified and partially characterized. They are useful in studying the contribution and function of the cAMP-pathway, as they separate the block in cAMP-pathway signaling from its usual cellular health defect; something that was previously only available if cells were supplemented with cAMP.

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