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MicroCommentary

Deciphering fungal dimorphism: Farnesol's unanswered questions

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Summary

Candida albicans **excretes *E,E*-farnesol as a virulence factor and quorum sensing molecule that prevents the yeast to hyphal conversion. Polke et al. (2016) identified *eed1*Δ/Δ as the first farnesol hypersensitive mutant of *C. albicans*. *eed1*Δ/Δ also excretes 10X more farnesol and while able to form hyphae, it cannot maintain hyphae. This mutant enables new research into unanswered questions, including the existence of potential farnesol receptors and transporters, regulation of farnesol synthesis, and relationships among farnesol, germ tube formation and hyphal maintenance. The *eed1* farnesol hypersensitivity can be explained by higher internal concentrations of farnesol or lower thresholds for response. One possibility invokes misexpression of a transporter. *Saccharomyces cerevisiae* and *C. albicans* have transporters for farnesylated peptides, like the a-factor pheromone, which could potentially also transport farnesol for virulence and quorum sensing. Significantly, these transporters are repressed in *MTLa/MTL*α *C. albicans*. An evolutionary pressure for *C. albicans* to become diploid could derive from its use of farnesol. Alternatively, maintenance of hyphal growth may increase the farnesol response threshold. Finally, Dpp1p, Dpp2p and Dpp3p are nonspecific pyrophosphatases responsible for farnesol synthesis. Changes in expression of these enzymes do not explain differences in farnesol levels implicating involvement of additional factors like a scaffolding molecule.**

The capacity to undergo changes in cell morphology is essential for the ability of many fungal pathogens to cause disease. *Candida albicans* is a serious opportunistic fungal pathogen of humans. In healthy people, it is a harmless member of the microbial flora in the gastrointestinal and urogenital tracts. However, when host defenses are compromised, it causes mucosal and disseminated infections that are often life-threatening. *C. albicans*, is able to grow in yeast and filamentous cell forms and, like many fungal pathogens, the ability to change between different cell forms is strongly correlated with its ability to cause disease.

In 2001, we identified the sesquiterpene *E,E*-farnesol as a quorum sensing molecule (QSM) produced by *C. albicans* (Hornby *et al.*, 2001). Throughout we will use farnesol for *E,E*-farnesol and define quorum sensing activity as the ability to block the yeast to hypha conversion in a cell density-dependent manner. However, farnesol has numerous other biological activities. Early on it was found that farnesol inhibited *C. albicans* biofilm formation (Ramage *et al.*, 2002), triggered apoptosis in other potentially competing fungi (Semighini *et al.*, 2006), and acted as a virulence factor in the mouse model of systemic candidiasis (Navarathna *et al.*, 2007). This latter finding raised the conundrum of how farnesol could act as a virulence factor when it also functioned *in vitro* to block germ tube formation (GTF) and hyphal growth, which are themselves essential for pathogenesis. This dilemma has been answered in part by the recent work of Hargarten *et al.* (2015) who showed that *C. albicans* white cells secrete farnesol as a chemoattractive stimulant of macrophages, a phagocyte that they are able to survive within and escape from. Farnesol, which is secreted by white cells only (Dumitru *et al.*, 2007), is a potent stimulator of macrophage chemokinesis; it results in an 8.5-fold increase in macrophage migration *in vitro* and a threefold increase in the peritoneal infiltration of macrophages *in vivo* (Hargarten *et al.*, 2015).

Since our publication in 2001, farnesol and its many roles in the biology of *C. albicans* have been of great interest to the *Candida* community, resulting in ca. 250 publications and 22 patents as of September 2016. However, despite this interest, 15 years later we still do

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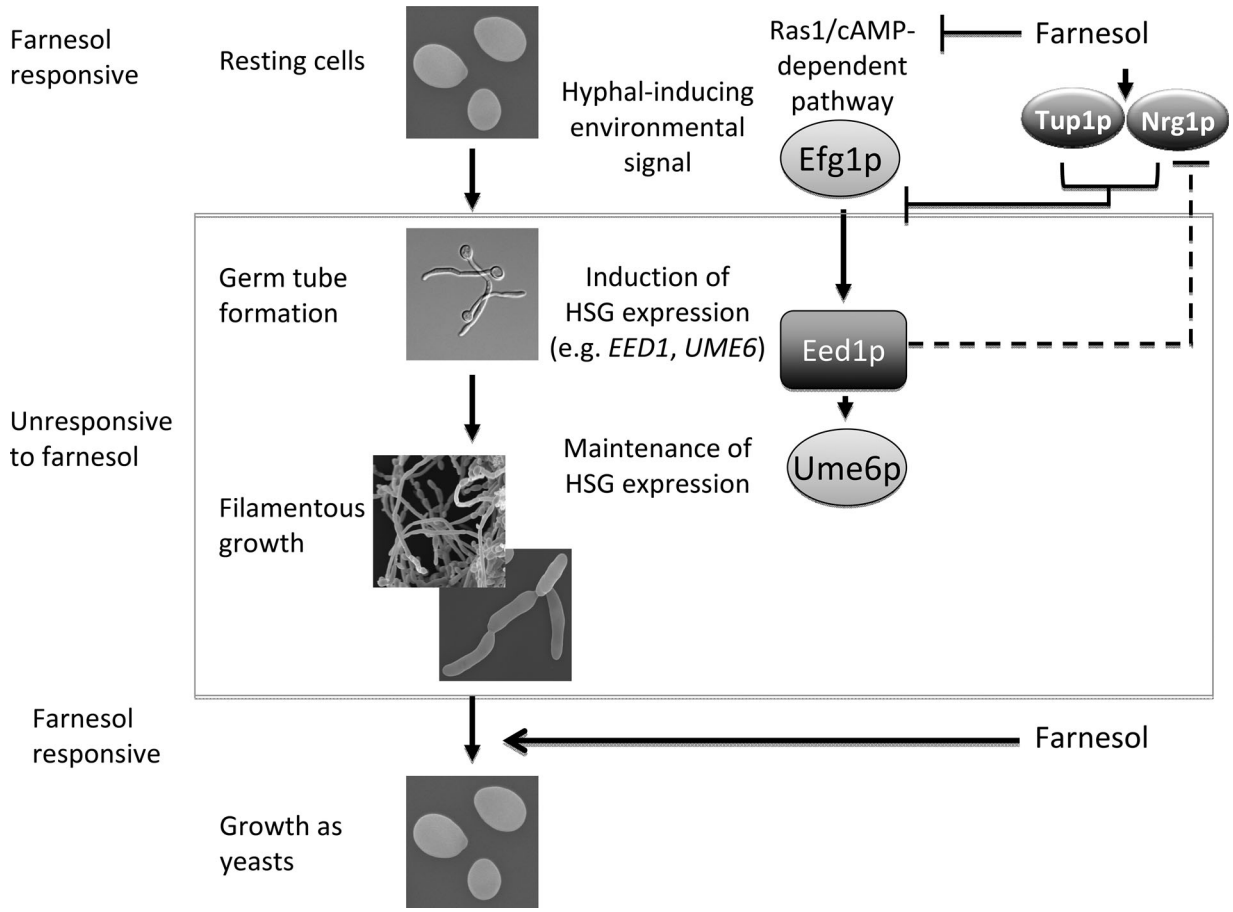

Fig. 1. The relationships between morphological development, related transcriptional changes, and farnesol signaling in *C. albicans*. Black lines represent active regulatory relationships while dotted lines represent potential indirect relationships. Transcription regulators are represented by ovals.

not know: 1/if there is a receptor/sensor for farnesol; 2/if there is a transport system for farnesol; 3/how farnesol synthesis is regulated [opaque and anaerobic cells turn off synthesis whereas *tup1* and *nrg1* mutants elevate synthesis 17–19 fold (Kebaara *et al.*, 2008); and 4/what the relationship is between farnesol and commitment. Commitment was defined by Mitchell and Soll (1979) and Chaffin and Wheeler (1981) for *C. albicans* and Muthukumar and Nickerson (1985) for *Ceratocystis ulmi* as the point in the events leading to germination or budding at which a cell may no longer choose. These organisms remain committed to a given morphology even in the absence of the original inducing conditions, i.e. resuspension in a growth medium which promotes the alternate morphology. In *C. albicans*, the yeast to hypha transition is well studied because of the synchrony inherent in starting with totipotent, single cells. In contrast, the transition from hyphae to yeasts is poorly studied, despite its evident importance in pathogenesis, e.g. the release of yeasts from biofilms as a reservoir for infection.

Differentiation from resting cells into filamentous cells can be broadly divided into four steps (Fig. 1): 1/resting cells in the G_0 phase of the cell cycle are totipotent and responsive to farnesol, 2/germ tube formation, unresponsive to farnesol, 3/hyphal or pseudohyphal growth, unresponsive to farnesol and 4/hyphal or pseudohyphal cells that respond to farnesol leading to budding of yeast cells. Experimentally we know that farnesol blocks the yeast to hypha conversion but does not block the elongation of preexisting hyphae, at least for a period of 6–10 h following GTF. This latter phenomenon is of direct relevance to morphogenesis and disease.

EED1 or epithelial escape and dissemination 1 regulates hyphal maintenance

EED1 (Epithelial Escape and Dissemination 1) is a unique *C. albicans* gene that is important for maintenance and persistence within the epithelium (Zakikhany *et al.*, 2007; Martin *et al.*, 2011). *EED1* expression is

sevenfold higher in hyphal-inducing conditions than in conditions favoring growth as yeasts. It is also upregulated in *C. albicans* in both a reconstituted human oral epithelium infection model and in samples from patients with oral candidiasis. It was first discovered by Zakikhany *et al*. (2007) who used the reconstituted human oral epithelium infection model to follow the establishment of oral candidiasis. Wild type *C. albicans* forms hyphae upon attachment to the epithelial surfaces, leading to invasion of the epithelial cells. The fungus then proliferates, disseminating deeper into the epithelial layers leading to cell damage and clinical symptoms of the disease. Like wild type *C. albicans*, mutants lacking *EED1* are able to form filaments and invade epithelial cells. However, these mutants then switch to yeasts that still proliferate intracellularly, but cannot escape from epithelial cells. As a result the mutants do not disseminate to other epithelial cells and thus cause reduced epithelial tissue damage (Zakikhany *et al*., 2007). *In vitro*, these *eed1* mutants initially form filaments in response to hyphal stimulants such as contact with plastic surfaces in RPMI with 10% serum at low cell densities (Zakikhany *et al*., 2007; Martin *et al*., 2011). However, hyphal elongation is always short lived and lateral yeast formation occurs remarkably quickly (Martin *et al*., 2011). Thus, the study of *EED1* and Eed1p allows the researcher to separate the initiation of hyphae from the extension of hyphae (Fig. 1). We take pleasure in noting that this distinction between cell division and cell elongation was the major theme in the work of W.J. Nickerson on yeast-mycelial dimorphism in *C. albicans* and other dimorphic fungi (Nickerson, 1948; Nickerson and Falcone, 1956). *EED1* likely encodes a positive regulator of hyphal extension. The availability of *eed1*Δ/Δ as a research tool overcomes one of the major difficulties in studying the hypha to yeast transition – its extended and non-synchronous time frame.

There is a caveat to the claim that *EED1* is unique in *C. albicans*. Maguire *et al*. (2013) showed that *EED1* was an example of a rapidly evolving gene, whose homology was revealed by a combination of BLASTP and synteny information, but not by BLASTP alone. Maguire *et al*. (2013) concluded that *EED1* was present in the common ancestor of the CTG clade, but that it is rapidly evolving and has undergone some particularly significant changes in the *C. albicans* lineage, probably associated with the ability of *C. albicans* to undergo true hyphal growth, a phenotype that is almost unique in the CTG clade. Thus, *EED1* may have a unique physiological function in *C. albicans*.

The molecular function of Eed1p is unknown, however it is important for expression of hyphal-specific or hyphal-associated genes (HSGs) during hyphal growth (Martin *et al*., 2011; Fig. 1). In wild type *C. albicans*, HSG expression is induced at the onset of germ tube formation and remains elevated during the subsequent hyphal growth (reviewed in Biswas *et al*., 2007; Whiteway and Bachewich, 2007). Hyphal-associated gene expression is also induced in *eed1*Δ/Δ at the onset of germ tube formation, but is not maintained (Martin *et al*., 2011). Instead expression of genes required for growth as yeasts, including *NRG1*, increases. The effect of Eed1p on HSG expression is largely mediated by Ume6p, a transcription factor, because ectopic expression of Ume6p rescues filamentation in an *eed1*Δ/Δ mutant and restores expression of HSGs (Martin *et al*., 2011; Fig. 1).

EED1 expression is regulated by transcription factors that are also involved in the response to farnesol (Fig. 1). The sevenfold increase in *EED1* expression in hyphal-inducing conditions is dependent on Efg1, the key transcriptional regulator of the Ras-cAMP signal transduction pathway (Martin *et al*., 2011). Efg1 is a transcriptional activator of hyphal-specific genes that is activated in response to environmental signals that induce hyphal growth. Eed1p functions downstream of Efg1p because ectopic expression of *EED1* rescues the filamentation defect of an *efg1* mutant (Martin *et al*., 2011). Similarly, *EED1* is repressed by Tup1p and Nrg1p because *EED1* expression is higher in *tup1*Δ/Δ and *nrg1*Δ/Δ mutants in conditions that favor both yeast and hyphal growth (Martin *et al*., 2011). Tup1p is a transcription repressor that negatively regulates the change from yeasts to hyphae by inhibiting hyphal-specific gene expression while Nrg1p is one of three DNA binding proteins that functions with the Tup1p complexes to inhibit gene expression. Farnesol blocks activation of the hyphal-specific genes regulated by Efg1 by inactivating the Ras-cAMP pathway and causes a ~2.5-fold increase in Tup1p expression (Davis-Hanna *et al*., 2008; Kebaara *et al*., 2008; Hall *et al*., 2011; Lindsay *et al*., 2012; Piispanen *et al*., 2013). Collectively, these interactions create a delicately poised feedback loop regulating cell morphology (Fig. 1).

Mutants altered in their response to farnesol

Polke *et al*. (2016) have now laid the foundation for a new way of addressing unanswered questions about farnesol function by examining several farnesol-related aspects of *EED1*. One approach to discerning farnesol's mode of action is to screen existing collections for *C. albicans* mutants which are altered in their response to farnesol. Langford *et al*. (2013) used this strategy to identify six mutants, including *czf1*, which were impaired in their farnesol response, i.e. they were still filamentous at 37°C in the presence of 50 μM farnesol. Now Polke

et al. (2016) have identified the first mutant (*eed1*Δ/Δ) that is hypersensitive to farnesol. This mutant was blocked in the yeast to hypha conversion by only 0.1 μM farnesol, making it 50–100 times more sensitive to farnesol than wild type *C. albicans*. The involvement of *EED1* in the response to farnesol is not surprising given its position in the hyphal induction mediated by the Ras-cAMP pathway (Fig. 1). What is surprising is that *eed1* is hypersensitive to farnesol because mutations in *TPK1* and *CZF1*, which encode a cAMP-dependent PKA and a transcription regulator of this pathway, respectively, both confer resistance to farnesol (Langford *et al*., 2013).

Polke *et al*. (2016) concluded that farnesol likely acts on *eed1* via a novel mechanism, quite distinct from its action as a QSM via inhibition of the RAS-cAMP signaling pathway (Davis-Hanna *et al*., 2008) or its ability to kill other yeasts and fungi by stimulating their mitochondria to produce excess ROS (Machida *et al*., 1998). Finally, and most intriguing, *eed1*Δ/Δ is not only hyper sensitive to farnesol but it also secretes more farnesol in a more rapid time frame. The hypersensitivity of *C. albicans eed1*Δ/Δ mutants to farnesol can be explained by either: 1/these mutants maintain a higher internal concentration of farnesol or 2/these mutants have a lower threshold for response to farnesol.

A regulated farnesol transporter?

An attractive explanation for these phenotypes (both hypersensitivity and hypersecretion) is that *eed1*Δ/Δ cells are able to transport farnesol more efficiently than wild type cells. This possibility would allow higher internal concentrations of farnesol at lower external concentrations. This mechanism could be either direct, via active transport or facilitated diffusion, or indirect via a major change in the lipid components of the cytoplasmic membrane. For instance, Ghannoum *et al*. (1990) reported that white cells of *C. albicans* had 4–7 times more sterols in their membranes than did opaque cells. Such major changes in sterol composition could alter the ease with which farnesol diffuses across these membranes. More sterols would make the membrane less fluid and thus less amenable to diffusion. Indeed, Dumitru *et al*. (2007) observed that three types of opaque cells, *C. albicans* WO-1, 3740 (*MTL*α/α), and 3745 (*MTL***a**/**a**), were lysed by 40 μM farnesol whereas the corresponding white cells remained 100% viable. Polke *et al*. (2016) addressed the possibility of secondary effects arising from changes in membrane lipid composition by showing that wild type and *eed1* mutants had equivalent sensitivity to lysis by the anionic detergent SDS, but it would still be nice to compare the actual membrane compositions.

Having a regulated farnesol transporter would provide an attractive explanation for several phenomena: 1/*eed1*Δ/Δ responds to lower levels of exogenous farnesol, Polke *et al*. (2016), because the farnesol no longer has to enter by diffusion. Similarly, the *eed1* mutant could excrete farnesol more rapidly because the farnesol no longer has to leave by diffusion. 2/*eed1*Δ/Δ secretes roughly 10X more farnesol than wild type *C. albicans* but the amounts of membrane-bound farnesol are much lower in *eed1*Δ/Δ (Polke *et al*., 2016) than in wild type (Navarathna *et al*., 2005). 3/Farnesol resistance or sensitivity is not an intrinsic feature of *C. albicans*. It can vary with the growth phase or other physiological changes. For instance, lag-and stationary-phase cells are not inhibited by farnesol concentrations up to 300 μM while exponential phase cells are inhibited by only 40 μM farnesol (Langford *et al*., 2010). This phenomenon had previously been described by Uppuluri *et al*. (2007).

These ideas could be tested by characterizing the uptake kinetics in both wild type and *eed1*Δ/Δ for radiolabeled or fluorescently labeled farnesol. It would be problematic to use a chemically modified farnesol such as biotinylated farnesol because the C-1 hydroxyl of farnesol is essential for its QSM activity. Additionally, Shchepin *et al*. (2003) found that all 40 of the natural and synthetic farnesol analogs they tested had less than 10% of the QSM activity of authentic farnesol. Changes as subtle as replacing a methylene with a sulfur or a methyl with an ethyl gave only 2-3% of the QSM activity. Instead, we designed and synthesized a fluorescent farnesol (Shchepin *et al*., 2005) which maintained the 3-dimensional structure of *E,E*-farnesol. Farnesol contains three non-conjugated carbon-carbon double bonds. We added two more double bonds between those three double bonds to make a fluorescent farnesol with five conjugated double bonds. *C. albicans* A72 cells stained with this fluorescent farnesol showed that the cytoplasmic membrane was stained along with an internal oval structure occupying ca. 1/4 of the cell volume (Shchepin *et al*., 2005). The identity of the internal structure is not known. No supplies of fluorescent farnesol are still available.

The a-factor mating pheromone is farnesylated

If the above analysis is correct, *C. albicans* may have a previously unrecognized, regulatable system that can transport farnesol. To identify such a transporter, we have thought about several approaches and candidates. The connection is not obvious since it has remained

undiscovered for the past 15 years. Is there homology with transport of insect juvenile hormone? Juvenile hormone is after all the 10,11 epoxide of farnesoic acid. Instead, is it one of the 214 membrane proteins identified by Cabezon *et al*. (2009) as the *C. albicans* plasma membrane proteome? Alternatively, we could look for candidate transporters among proteins already known to exhibit specificity for farnesol (C_{15}) or geranylgeraniol (C_{20}), such as farnesol transferase (FTase) or geranylgeranyl transferase 1 (GGTase) (Kelly *et al*., 2000). Both enzymes are zinc-dependent heterodimers comprising an α and a β subunit, which prenylate proteins by attaching farnesol or geranylgeraniol, respectively, in a thioether linkage to the cysteine of a CaaX motif at the C-terminus. The α subunit (Ram2p) is shared by GGTase and FTase in both yeasts and mammals while the β subunits for *C. albicans* are Cdc43p and Rho3p for GGTase and FTase respectively. Significantly, Rho3p (orf19.3534) is one of the proteins in the *C. albicans* plasma membrane proteome (Cabezon *et al*., 2009).

Relevant farnesol specific domains should also be present in a transporter able to import or export a farnesylated protein or peptide. We were drawn to the fact that both *S. cerevisiae* and *C. albicans* secrete a farnesylated peptide mating pheromone. This farnesylated peptide has to be secreted by **a** mating type cells and then recognized and imported by α mating type cells, by Ste6p and Ste3p respectively. Ste3p and Ste6p should both have farnesol-specific portions of their peptide binding sites so that they can distinguish farnesylated peptides from unmodified peptides. In this model, Ste3p or Ste6p would transport free farnesol as well as the farnesylated peptide. Ste6p (orf 19.7440) is an ATP binding cassette (ABC) transporter that exports the **a**-factor peptide. It is not regulated during white opaque switching and Ste6p is constitutively transcribed. In contrast, Ste3p (orf19.2492) is the **a**-factor receptor on α cells. Expression of *STE3* is increased 300–1000 fold in opaque cells. A key point is that in *MTL**a**/α* heterozygotes, both a-specific and α-specific gene expression is turned off. This model is attractive in that it provides a ready explanation for the farnesol sensitivity of aerobically grown opaque cells. The white cells are typical budding yeasts, usually *MTL**a**/α*, produce lots of farnesol, and are resistant to up to 300 μM farnesol. In contrast, opaque cells are elongated, either *MTL**a**/**a*** or *MTLα/α*, do not secrete farnesol, and are as sensitive to farnesol as are *S. cerevisiae* and other fungi. Dumitru *et al*. (2007) showed that aerobically opaque cells of *C. albicans* were lysed quickly by 20-40 μM farnesol. This observation is consistent with either Ste3p or Ste6p being expressed in the opaque cells, with their presence being responsible for the dramatic farnesol sensitivity of opaque cells. However, *eed1*Δ/Δ does not fit the scenario. Neither *STE3* nor *STE6* was altered in expression (Martin *et al*., 2011). It is possible that another as yet unidentified farnesol transporter is upregulated in *eed1*Δ/Δ and, as pointed out by Polke *et al*. (2016), with *eed1*Δ/Δ as a model for farnesol hypersensitive mutants we may now discover several more examples of this unusual phenotype.

A potential source of evolutionary pressures for *C. albicans* to be a diploid

Why is *C. albicans* diploid? For many years it was thought that *C. albicans* had to be diploid because it carried many recessive lethal mutations. This idea was replaced by the seminal work of Hickman *et al*. (2013) who isolated a series of haploid *C. albicans* cells. They showed that the haploid cells were smaller than the diploid cells and they grew more slowly. However, they were still able to undergo yeast-hyphal and white-opaque switching, form both pseudohyphae and chlamydospores, and mate [if haploid opaques of opposite mating type were present]. Hickman *et al*. (2013) concluded that haploid formation was not a rare event at all. However, haploids only constitute a very small % of the total population because of their low competitive fitness relative to that of heterozygous diploids. Hickman *et al*. (2013) did not define the basis of that low competitive fitness but increased sensitivity to farnesol is certainly a possibility. Six *MTL**a*** and five *MTLα* haploid cell lines are available (Hickman *et al*., 2013) to assess the farnesol concentrations needed to achieve cell lysis or to block hyphal growth. Are one or both of the haploid mating type **a** or α strains hypersensitive to farnesol? If so, it suggests the evolutionary codicil that the drive to diploid status for *C. albicans* coincided with the drive to overproduce and secrete farnesol. It also suggests that the diploid *S. cerevisiae* BY4743 (*MTL**a**/α*) should be significantly more resistant to exogenous farnesol than the isogenic haploid *S. cerevisiae* BY4741.

The connection between farnesol and the diploid status of *C. albicans* also fits the work of Lockhart *et al*. (2005). Farnesol is a known virulence factor for *C. albicans* (Navarathna *et al*., 2007; Hargarten *et al*., 2015). Lockhart *et al*. (2005) injected mice with *MTL**a**/**a***, ***a**/α*, or *α/α* strains of *C. albicans*. Their single-strain injection experiments showed that the *MTL**a**/α* strains were far more virulent than either the *MTL**a**/**a*** or *MTLα/α* strains. Similarly, when equal numbers of parent and daughter cells were co-injected, *MTL**a**/α* always exhibited a competitive advantage. Lockhart *et al*. (2005) proposed that heterozygosity at the *MTL* locus repressed white-opaque switching and the genes involved in the mating process, but it also affected virulence, providing a

competitive advantage to the *MTL***a**/α genotype that conserves the mating system of *C. albicans* in nature. We now suggest that farnesol may provide the mechanism connecting the *MTL* locus with virulence.

A decreased threshold for response to farnesol?

Resting cells are in the G_0 phase of the cell cycle and environmental inducers of hyphal growth will trigger germ tube formation (GTF) (Fig. 1). The addition of farnesol to resting cells inhibits GTF if added at any time up until the actual emergence of germ tubes (Hornby *et al*., 2001; Mosel *et al*., 2005). These cells will remain as yeasts. However, once a germ tube has emerged, *C. albicans* is no longer able to respond to farnesol (Mosel *et al*., 2005). *C. albicans* will typically maintain hyphal or pseudohyphal growth through multiple cell divisions. During this phase, cells remain unresponsive to farnesol. At some still poorly defined point, cells regain the ability to switch from hyphal to yeast growth and once again respond to farnesol (Fig. 1).

Many environmental inducers of hyphal development initiate signal transduction by the Ras-cAMP pathway leading to a change in transcriptional regulation (Hogan and Sundstrom, 2009; Inglis and Sherlock, 2013). Initiation of hyphal development depends on expression of the HSGs, a process that involves both release from negative regulation by Tup1p and Nrg1p as well as activation of HSGs by transcription factors including Efg1p (reviewed in Liu, 2001). Sustained hyphal growth depends on continued HSG expression. This is accomplished, in part, by expression of the transcription factor Ume6p and chromatin remodeling of HSG promoters to prevent binding of Nrg1p (reviewed in Lu *et al*., 2014a).

Farnesol blocks hyphal development in at least three ways: 1/it inhibits the Ras-cAMP signal transduction pathway (Davis-Hanna *et al*., 2008; Hall *et al*., 2011), 2/it causes a 2.5-fold increase in Tup1p levels (Kebaara *et al*., 2008), And 3/it blocks the Sok1-mediated degradation of Nrg1p (Lu *et al*., 2014b). The observations of Polke *et al*. (2016) that the hypersensitivity of *eed1*Δ/Δ mutants is independent of both the Ras-cAMP pathway and Nrg1p suggests that the ability to transition from initiation of HSG expression to maintenance of HSG is important for farnesol sensitivity. This finding is unexpected because wild type *C. albicans* become unresponsive to farnesol at the time when germ tubes first emerge, which is prior to the transition to maintenance of HSG expression (Mosel *et al*., 2005). This juxtaposition also implies that the different cellular responses to farnesol are additive so that loss of the ability to maintain HSG expression lowers the threshold for a cell's response to farnesol. Thus the *eed1*Δ/Δ mutant provides a new opportunity to study the relationships among maintenance of hyphal gene expression, the response to farnesol during filamentous growth, and the transition from filamentous to yeast growth.

Why is farnesol synthesis elevated in *tup1*, *nrg1* and *eed1* mutants?

Farnesol is synthesized from farnesyl pyrophosphate (FPP) by the lipid pyrophosphatases Dpp2p and Dpp3p (Navarathna *et al*., 2007) and possibly also Dpp1p (Ganguly *et al*., 2011, Polke *et al*., 2016). The amounts of farnesol produced are roughly proportional to the mass of *C. albicans* cells grown aerobically in a defined glucose-phosphate-proline medium at all temperatures tested from 23 to 43°C (Hornby *et al*., 2001). However, farnesol synthesis can be regulated: 1/Synthesis is shut off during anaerobic growth (Dumitru *et al*., 2004); 2/Farnesol is synthesized by white cells but not by opaque cells (Dumitru *et al*., 2007); 3/*tup1* and *nrg1* mutants of *C. albicans* overproduce farnesol 19- and 17-fold, respectively (Kebaara *et al*., 2008); 4/*eed1* mutants overproduce farnesol 10-fold (Polke *et al*., 2016) and 5/*zap1* mutants produce sixfold less farnesol (Ganguly *et al*., 2011). The challenge in discerning how farnesol is regulated is evident from the fact that *tup1* and *nrg1* mutants are locked in filamentous growth, whereas *eed1* mutants cannot maintain filamentous growth and are hypersensitive (0.5 uM) to farnesol.

It is unlikely that farnesol regulation is primarily at the level of *DPP2* and *DPP3* expression. *DPP2* and *DPP3* mRNA levels are not affected by loss of *TUP1* or *NRG1* or by growth of *C. albicans* in the white or opaque phases, producing high and no farnesol, respectively (Kadosh and Johnson, 2005; Hargarten and Atkin, unpublished; Kebaara *et al*., 2008). And farnesol production levels were reduced ca. 85% when *DPP3* was knocked out in an auxotrophic SN152 background (Kadosh and Johnson, 2005; Navarathna *et al*., 2007) but remained unchanged, still high, in an *eed1* background (Polke *et al*., 2016).

Several mechanisms for regulating farnesol production are possible. First, regulation via carbon flow through the ergosterol biosynthetic pathway, with carbon flow determining the FPP pool size. Does the FPP pool size exceed the Km values for Dpp1p, Dpp2p, and Dpp3p? We suggested this explanation for our observations that sublethal levels of zaragozic acid (Hornby *et al*., 2003) and four azole antibiotics (Hornby and Nickerson, 2004) caused 10- to 40-fold increases in farnesol production by *C. albicans*. Presumably, blocking the carbon flow to ergosterol led to the accumulation of precursor molecules including FPP. As another example, there is no cell

density or inoculum size effect when *C. albicans* A72 cells are triggered for GTF by N-acetylglucosamine (GlcNAc); similar rates were observed with inocula of 10^5 to 10^8 cells/ml (Hornby *et al*., 2003). This observation was perplexing because the discovery of farnesol as a QSM was driven by the study of cell density effects in fungal dimorphism. However, the observation made sense when viewed in the context that GlcNAc-induced GTF is a differentiation, not growth, and sterol biosynthesis has not been turned on during that differentiation (Sundaram *et al*., 1981). Thus, an indicated experiment would be to measure the time course of farnesol production, if any, during germination as induced by N-acetylglucosamine versus a complete growth medium such as mGPP, Spider medium, or Lee's medium. In any case, measurement of the FPP pool sizes could usefully accompany future reports on farnesol production levels.

Second, excreted farnesol is in equilibrium with both internal and membrane bound farnesol (Navarathna *et al*., 2005). Maintenance of this equilibrium is nicely exploited by industrial protocols that provide an external lipid sink such as mineral oil to maximize the microbial production of farnesol (Muramatsu *et al*., 2002). If diffusion across the cytoplasmic membrane is the rate limiting step, as seems likely, then farnesol production would be facilitated by introducing a farnesol transport protein, as may have occurred in the *eed1* mutant characterized by Polke *et al*. (2016).

Third, many layers of regulation occur after gene expression. The presence or absence of FPP pyrophosphatase activity could be determined by whether the relevant mRNA are translated, whether Dpp2p and Dpp3p are activated/inactivated by post-translational modifications, or whether they are targeted for proteolysis by interactions with farnesol or geranylgeraniol pyrophosphate in the same manner by which HMG CoA reductase is regulated in *S. cerevisiae* (Shearer and Hampton, 2005).

Finally, Dpp1p, Dpp2p, and Dpp3p are rather non-specific pyrophosphatases, active on many lipid pyrophosphates (Faulkner *et al*., 1999). Thus, they have substrates and products other than FPP and farnesol, and the *dpp1, dpp2*, and *dpp3* mutants should have complex phenotypes. The specificity regarding which lipid pyrophosphate is the actual substrate may be provided by the presence of a scaffolding protein or RNA which delivers the intended substrate to the pyrophosphatase, or whose presence is necessary for the pyrophosphatase to act as a functional α/β dimer.

Future directions

We can use the *C. albicans eed1*Δ/Δ mutant as a tool to investigate the remaining questions on the role of farnesol in fungal dimorphism. It is the first mutant that is hypersensitive to farnesol; does more farnesol enter the fungal cell or is less farnesol now sufficient to accomplish the observed changes? Also, we now have a new tool to separate the initiation of hyphae (GTF) from the maintenance of hyphae. Is chromatin remodeling a necessary step for either GTF or hyphal maintenance? In both cases, answers may be gleaned, in part, from mining the gene expression data for the *eed1*Δ/Δ mutant (Martin *et al*., 2011). They compared *eed1*Δ/Δ versus its wild type parent after 1, 12, or 24 h growth on plastic, and identified 910 genes that were at least two-fold differentially expressed in the mutant at one of the time points; 441 were down-regulated and 469 were up-regulated. *STE3* and *STE6* were not among the genes that were up-regulated (alas). In particular, many of the hyphal associated genes were down-regulated after 12 or 24 h while *NRG1* and *WOR2* were up-regulated (Martin *et al*., 2011). These observations suggest a feedback loop where *EED1* and *UME6* are repressed by Nrg1p while expression of *EED1* and *UME6* is required for exclusion of Nrg1p from the HSG promoters (Fig. 1). These gene expression data sets provide a fertile resource for answering these questions, with the caveat that the relevant gene may be expressed at such a low level it isn't in the data set or it would have been detected at some time point between 1 and 12 h. In the process, we may also discover the mechanism whereby excess biotin (4 uM) stimulates GTF (Lee *et al*., 1975; Ahmad Hussin *et al*., 2016) as well as histone biotinylation (Hasim *et al*., 2013) in *C. albicans*. Are the two phenomena causally related, possibly via chromatin remodeling? The response of *eed1*Δ/Δ to biotin has yet to be investigated.

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