New surprises from within the black box of fungal melanization

Alexander Idnurm
Division of Cell Biology and Biophysics; School of Biological Sciences; University of Missouri at Kansas City; Kansas City, MO USA

Key words: Cryptococcus neoformans, gene expression, melanin, microarray, quorum sensing

Melanins represent a group of dark pigments made of polymerized phenolic and indole subunits and whose exact structures are undefined. Melanin plays a number of roles in the biology of fungi, including being essential for pathogenesis via several mechanisms. Plant pathogens use melanin to build rigid appressoria structures to penetrate plant cell walls while human pathogens use it to protect against host immune responses. Despite this requirement for pathogenesis in diverse organisms, we remain in the dark about many features of melanin. In this issue of Virulence, Eisenman et al.2 reveal some surprising new aspects about melaninization in the human pathogenic fungus Cryptococcus neoformans.

C. neoformans and C. gattii are pathogens found around the world, causing disease in both immunocompromised and healthy patients, as well as in diverse mammalian species that include domestic pets, livestock, marsupials and even porpoises. The predominance of fatal cases is in AIDS patients in sub-Saharan Africa.2 The sudden emergence of C. gattii on Vancouver Island and subsequent spread into mainland Canada and the United States during the last decade, causing disease in healthy people and their pets, further illustrates the complex health challenges posed globally by this pair of species.3 Cryptococcus is contracted by inhalation to initiate a pulmonary disease in susceptible individuals. The fungus spreads from the lungs throughout the body, with the unusual ability to cross the blood-brain barrier to cause fatal meningitis.4 C. neoformans requires phenolic substrates to produce melanin. Those substrates are most prevalent in the brain, and melanized C. neoformans cells can be visualized in brain samples from infected people using immunofluorescence microscopy or isolated as melanin “ghosts” by boiling tissue in hydrochloric acid.4 This latter technique highlights just how resistant melanin is, accounting for its protective properties against stresses that fungi encounter in the wild or during infection.

One the hallmarks to identify C. neoformans is its production of melanin in response to external substrates. C. neoformans cannot make melanin de novo from basic carbon and nitrogen sources, thus distinguishing it from fungi that can do so. This property led to a facile isolation medium made of ground seeds of Guizotia abyssinica (niger seed agar) on which Cryptococcus colonies turn brown to black.5 Beyond being an essential clinical diagnostic tool, C. neoformans melanin has been extensively studied because it is required for full virulence. The central enzyme, laccase, is absolutely required for formation of the pigment since mutation of the LAC1 gene abolishes melanin production.6 Concomitantly, laccase mutants are attenuated for virulence.6,9 Although the lac1 mutant cells establish a robust infection in the lungs, they have limited ability to colonize the brain.10,11

Cryptococcus also serves as a model for understanding melanin properties more generally. For example, an antibody raised against Cryptococcus melanin recognizes mammalian melanin. Coupling a radioisotope to this antibody enables targeting and treatment of melanoma, an application currently in clinical trials.11 Thus, investigating Cryptococcus melanin has impacts beyond the field of infectious disease.

Despite extensive research on Cryptococcus melanin, numerous questions remain about how the fungus produces melanin, including what signals are sensed, the physical properties of the melanin(s) made, the substrates used within the host or the environment, and how the fungus manages to remodel the melanin shell during growth and cell division. In the current study, two striking new observations are made that extend beyond these fundamental questions.

The first surprise is the small number of genes regulated by a substrate, L-DOPA, to make melanin. Exposure of C. neoformans to a range of L-DOPA concentrations revealed an optimum at which melanization would occur. High concentrations reduce growth rate and also melanin production. Eisenman et al. used a complete genome microarray to measure transcript levels 4 h after adding L-DOPA to cultures, at a time point when the cells become pigmented. A mere eight genes were induced as detected on the microarray. It is not clear what function these genes play in melanization since none had been previously investigated, but they clearly represent targets for mutation studies in the future. Many of the genes are predicted to relate to responding to stress, suggesting that either the substrate or the melanization process is harmful to the fungus and consistent with reduced growth at higher concentrations of L-DOPA. Curiously, the induction of five of the genes in response to L-DOPA is abolished in a laccase mutant strain. This indicates that their regulation relies on the biosynthesis of melanin, rather than directly on the substrate. The natural substrates that C. neoformans uses to make melanin in
the wild or during infection are unknown, although there are candidate molecules for within the central nervous system and in the environment. One hypothesis that can be based on the current study is that the substrates within the brain are detrimental to fungi, and that melanization in *C. neoformans* has a detoxification benefit in addition to the established function in protecting against a diverse set of external stresses.

It is worth contrasting the handful of genes induced by L-DOPA in *C. neoformans* with expression analyses of other factors required for pathogenesis. The shift to mammalian body temperature causes hundreds of genes to change in transcript abundance, as known from the earliest genome-level comparisons that used partial genome data, covering a fraction of the ~6,500 genes in the genome. More recent studies have found that thousands of genes are transcriptionally regulated by different conditions or certain genes. Most approaches to examine changes in expression have measured transcript abundance. However, how closely transcription is coupled to translation in *C. neoformans* largely remains to be elucidated. In at least one process, that of RNA interference, the Ago1, Dcr1 and Rpd1 proteins are upregulated during mating whereas their corresponding transcripts are not. For melanin, the authors have previously shown that laccase is preformed in vesicles ready for delivery outside the cell upon inducing conditions. Melanization under inducing conditions would not require a transcriptional response, thereby explaining the paucity of genes found from the microarray analysis. On the other hand, other experiments show that the laccase gene is also regulated at the transcript level, indicating that a full gamut of regulatory steps influences melanization. As such, the biosynthesis of melanin could be developed as a system to tease out connections between transcription and functional end product in *C. neoformans*. However melanization is regulated, the small number of genes induced by L-DOPA is nevertheless a surprising observation because of the wide-ranging effects melanin has on the fungal cell and that at higher concentrations L-DOPA inhibits growth.

The second surprise is the effect of culture age and cell density on melanization. Aged *C. neoformans* cells become melanized more rapidly that youthful cells, while higher cell densities in liquid culture or on agar plates produce more melanin. Recent studies have begun to investigate the effects of age on *C. neoformans* cells to reveal other effects, like an increase in phenotypic switching frequency. Nevertheless, most researchers use a standard overnight culture of cells grown in rich medium for starting inocula in experiments, a condition that is unlikely to reflect what occurs in nature or before a person becomes infected. The cell density effect is reminiscent of quorum sensing, a process by which microbes measure cell density through secreted molecules to trigger a response at critical concentration thresholds. Here, the effects of cell density cannot be explained by a simple model of depletion of nutrients regulating melanization. Addition of other fungi to plates of *C. neoformans* inoculated at a low cell densities can enhance the rate of melanization, but not to the same level as seen at high cell densities (Fig. 1). Quorum sensing is a well-characterized property of some bacterial species, but less well explored in the fungi beyond the pathogen *Candida albicans*. However, an effect like quorum sensing on cell growth has also been detected in *C. neoformans* that features a small 11 amino acid secreted peptide, but only in strains with mutations in the *TUP1* gene and in a subset of strain backgrounds. The links between these cell density effects, if any, are unknown.

The discovery that melanization depends on age and cell density has implications for pathogenesis and detection of the species. For survival of cells in a population, one would predict that old cells and/or those found within dense cultures would be at a disadvantage compared to young cells actively dividing in ample media conditions. However, Eisenman et al. show that those seemingly non-favorable...

---

**Figure 1.** Cell density affects melanization of *Cryptococcus neoformans*. L-DOPA agar plates inoculated with higher colony forming units (CFUs) of strain JEC21 are more pigmented (A) than those with fewer CFUs (B). Co-inoculation with another basidiomycete yeast, *Sporobolomyces* sp. strain IAM 13481, whose colonies produce pink carotenoid pigments that distinguish them from colonies of *C. neoformans*, does not rescue the level of melanization of *C. neoformans* at low cell densities to that of high densities (C). The three *C. neoformans* colonies on the plate are marked by arrows. See also Figure 3 of Eisenman et al.
conditions act to turn on melanization, enhancing survival and potentially virulence if those cells are inhaled by people. Further, an increase in phenotypic switching rates in aged cells could also provide better adaptability. A prediction from these findings is that growth conditions may influence the outcome of an infection. This has been demonstrated independently, in other studies not focused on melanin, in which the culture conditions used prior to inoculating the cells into animals influences the rapidity of disease development. A second prediction is that environmental or clinical samples from older and denser sources of Cryptococcus neoformans would melanize better when plated. This could lead to skews in the detection and subsequent characterization of cells from within a population and warrants further investigation on media types used for environmental sampling or clinical diagnosis.

The research by Eisenman et al. raises numerous directions for future studies. For example, what signal and pathway controls the transcriptional response to melanization, and are more extensive transcriptional differences to be observed at later time points under melanizing conditions? Perhaps the most immediate direction is to elucidate the underlying mechanisms by which age or cell density are sensed in Cryptococcus neoformans. Will this represent a system like quorum sensing, with a sharp demarcation at a particular cell density, or a gradient of response? The use of melanization, with the ability both for rapid “black-and-white” scoring and for accurate quantification, coupled to the experimental strengths of Cryptococcus neoformans, offers a rapid way to gain insights into density-dependent responses in fungi in general.

In closing, melanin remains an enigmatic substance across biology. These studies illustrate that the response of pathogens to their environments to induce melanin production is even more complex than previously thought.