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Marcio L. Rodrigues *Editor*

Fungal Physiology and Immunopathogenesis

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Fungal Physiology and Immunopathogenesis

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Preface

Fungal diseases have been neglected for decades, which has negatively impacted the expansion of Medical Mycology and the rate of knowledge generation in this field. However, an unacceptably high number of deaths due to fungal infections (1.5 million people every year) has stimulated an appreciable expansion of Medical Mycology. Regrettably, the unquestionable progress achieved during the last two decades is still insufficient to place fungal infections at the level of knowledge generation and innovation that is observed for other infectious diseases. For instance, there are no licensed antifungal vaccines. Treatment of fungal diseases is unaffordable for millions of patients living under socio-economical restrictions. When available, antifungal treatment is expensive and associated with many undesirable side effects. The most recent antifungal drug introduced into clinics is now 18 years old. We are now facing the unexpected emergence of multidrug-resistant fungal pathogens and the alternatives to fighting this problem are very limited. This complex scenario reveals our unpreparedness to deal with clinical conditions deriving from fungal infections. These problems are likely a consequence of reduced funding for fungal research, which is much lower than that available for diseases of similar impact to human health. In summary, even with the expansion of Mycology and great scientific contributions in the last decades, it is clear that the field of fungal diseases demands more research and consequently accelerated the generation of knowledge and innovation.

The great scientific advances resulting in more sophisticated and comprehensive methods for the analysis of biological questions related to human health has positively impacted Medical Mycology research. Unquestionably, the methodological advances developed in Genetics, Immunology, Systems Biology, and Cell Biology have improved our understanding on how fungal pathogens interact with the host, resulting in the generation of damage to host tissues or the control of fungal infections. In this context, this volume efficiently illustrates the progress of

knowledge generation in Medical Mycology. We invite the reader to visit the recent findings showing how fungal cells dynamically respond to different stimuli to cause damage to host cells or to adapt to different microenvironments related to disease progress or control.

Curitiba, Brazil Marcio L. Rodrigues

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The Role of Melanin in Fungal Pathogenesis for Animal Hosts

Daniel F. Q. Smith and Arturo Casadevall

Contents

Abstract Melanins are a class of pigments that are ubiquitous throughout biology. They play incredibly diverse and important roles ranging from radiation protection to immune defense, camouflage, and virulence. Fungi have evolved to use melanin to be able to persist in the environment and within organisms. Fungal melanins are often located within the cell well and are able to neutralize reactive oxygen species and other radicals, defend against UV radiation, bind and sequester non-specific peptides and compounds, and produce a physical barrier that defends the cell. For this reason, melanized fungi are often well-suited to be human pathogens—melanin allows fungi to neutralize the microbicidal oxidative bursts of our innate immune system, bind and inactivate to antimicrobial peptides and enzymes, sequester antifungal pharmaceuticals, and create a shield to block immune recognition of the fungus. Due to the importance and pervasiveness of melanin in fungal virulence,

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mammalian immune systems have evolved antifungal strategies that involve directly detecting and binding to fungal melanins. Such strategies include the use of melanin-specific antibody responses and C-type lectins like the newly discovered melanin-specific MelLec receptor.

1 Diversity of Fungal Melanins

Melanins are found in all biological kingdoms where they perform fundamental roles in the survival of organisms. In the microbial world, melanin pigments have been associated with numerous functions including energy harvesting and survival in stressful environments (Casadevall et al. 2017; Gessler et al. 2014). Broadly speaking, melanins are a class of black, brown, red, and yellow pigments that have similar characteristics such as: resistance to acid hydrolysis, amorphous structures, form through a catecholic, phenolic, or indolic precursor, insolubility in water, negative charges have broad monotonic absorbance spectra, and a stable free radical structure. Despite these similar characteristics, melanins are a highly heterogenous lot, with regards to structure, composition, and functional use.

There are several different classes of melanins, each produced through different biosynthetic pathways including: black–brown eumelanin produced by catecholamine oxidation and reactive quinone intermediates, and the polymerization of subsequently produced indoles, red–yellow pheomelanin produced by the reaction of oxidized catecholamines with cysteine-containing amino acids, light-brown pyomelanin produced through tyrosine catabolism and polymerization of homogentisic acid, and black–brown DHN melanin produced from acetyl-CoA and the polyketide synthesis pathway. The predominant melanins found in the fungal kingdom are DOPA melanins and DHN melanins. In addition to these common fungal melanins, there is evidence that some fungi produce pyomelanins, resulting from the breakdown of aromatic amino acids, particularly tyrosine. A partial list of melanized fungi, the type of melanin(s) they produce, and the enzymatic pathway(s) are summarized in Table 1.

In this essay, we focus on the role of melanin on fungal pathogenesis in animals. We note that melanin also has important in fungal pathogenesis for plants, particularly in fungal invasion and penetration into the plant through turgor pressure buildup. Interested readers may consult other reviews and papers such as (Chen et al. 2004; Howard and Valent 1996; Martin-Urdiroz et al. 2016; Ryder and Talbot 2015). Further, we note that there have been previous reviews on the role of melanin on fungal pathogenesis (Chowdhary et al. 2014; Gómez and Nosanchuk 2003; Jacobson 2000; Nosanchuk and Casadevall 2003; Revankar and Sutton 2010; Seyedmousavi et al. 2014) which provide additional perspectives.

Table 1 Melanin and melanin biosynthesis of selected fungi

Species	Fungal form	Melanin type	Enzymes and pathways	References
Aspergillus flavus	Conidia	DOPA	Tyrosinase	Inamdar et al. (2014), Pal et al. (2014)
Aspergillus fumigatus	Conidia	DOPA, DHN, Pyo	Laccase, PKS (*alb1, arp1, arp2*), TDP (*hhpD, hmgA*)	Schmaler-Ripcke et al. (2009), Tsai et al. (1999), Youngchim et al. (2004)
Aspergillus nidulans	Conidia	DOPA	Tyrosinase, Laccase	Bull (1970a, b), Bull and Carter (1973), Gonçalves et al. (2012), Kurtz and Champe (1982)
Aspergillus niger	Conidia	DOPA, DHN	Unknown, PKS (*albA, aygA*)	Chiang et al. (2011), Pal et al. (2014)
Candida albicans	Yeast	DOPA	Unknown/ Laccase	Morris-Jones et al. (2005)
Cryptococcus neoformans	Yeast	DOPA, Pyo	Laccase (*CNLAC1, CNLAC2*)	Frases et al. (2007), Shaw and Kapica (1972), Williamson (1994)
Cryptococcus gattii	Yeast	DOPA	Laccase	Chan and Tay (2010)
Exophiala (Wangiella) dermatitidis	Yeast Hyphae	DOPA, DHN DHN	Laccase; PKS (*WdPKS1*) PKS (*WdPKS1*)	Dixon et al. (1992), Geis, et al. (1984), Feng et al. (2001), Paolo et al. (2006), Wheeler et al. (2008)
Fonsecaea pedrosoi	Conidia Hyphae	DHN DHN	PKS PKS	Cunha et al. (2005)
Histoplasma capsulatum	Yeast Condia	DOPA, Pyo DOPA, DHN	Laccase, TDP PKS? Laccase?	Almeida-Paes et al. (2018), Nosanchuk et al. (2002)
Lomentospora prolificans	Conidia	DHN	PKS (*PKS1, 4HNR, SCD1*)	Al-Laaeiby et al. (2016)
Paracoccidioides brasiliensis	Yeast Conidia	DOPA DHN?	Laccase PKS?	Gómez et al. (2001)
Sporothrix schenckii	Yeast Conidia Hyphae	DOPA, Pyo DHN, DOPA, Pyo DOPA, Pyo	Laccase, TDP PKS, Laccase, TDP Laccase, TDP	Almeida-Paes et al. (2009), Almeida-Paes et al. (2012), Morris-Jones et al. (2003), Teixeira et al. (2010)
Talaromyces (Penicillium) marneffei	Yeast Conidia/ Hyphae	DOPA, DHN, Pyo DHN	Laccase, PKS (*alb1, arp1, arp2*), TDP (*hpdA, hmgR*) PKS (*alb1, arp1, arp2*)	Boyce et al. (2015), Kaewmalakul et al. (2014), Liu et al. (2014), Youngchim et al. (2005), Woo et al. (2010)

Abbreviations DOPA melanin (DOPA), DHN Melanin (DHN), Pyomelanin (Pyo), Polyketide Synthase Pathway (PKS), Tyrosine Degradation Pathway (TDP)

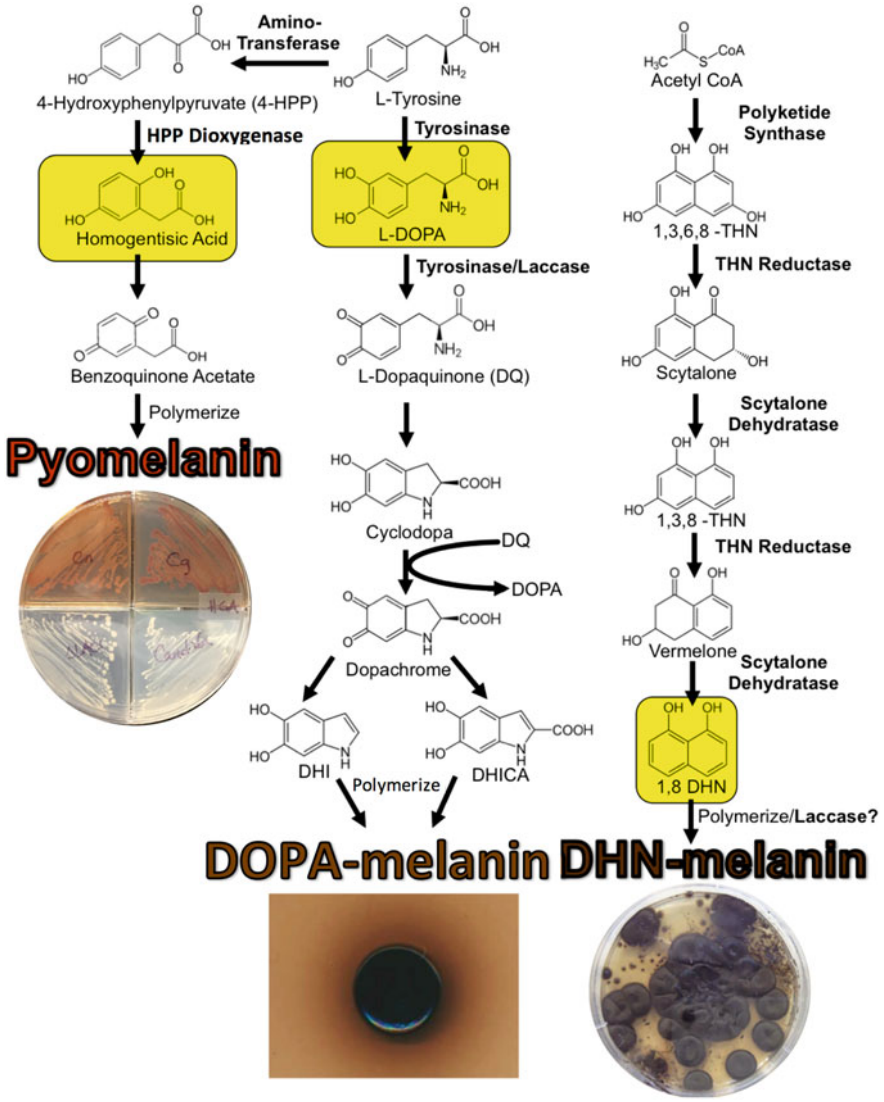
1.1 Characteristics and Synthesis of DOPA-Melanin

DOPA melanin is a highly common black–brown form of melanin that is typically produced from the hydroxylation of tyrosine, and the oxidation of catecholamines, such as L-DOPA (3,4-dihydroxyphenylalanine) and dopamine, into quinones (García-Borrón and Sánchez 2011). The quinones are highly reactive and spontaneously cyclize, undergo further oxidation, and inevitably form indoles such as dihydroxyindole (DHI) and dihydroxyindole carboxylic acid (DHICA). DHI and DHICA polymerize into eumelanin, forming amorphous, hydrophobic, black and brown polymers (Fig. 1). The Raper-Mason pathway of melanin biosynthesis best typifies this process, uncovered in the first half of the twentieth century (Mason 1948; Raper 1927).

While the oxidation of catecholamines, resulting in inevitable formation of dark pigments, can occur spontaneously at room temperature and following light exposure, organisms typically use enzymes to catalyze and control this reaction. Two classes of enzymes, laccases (EC 1.10.3.2) and phenol oxidases (EC. 1.14.18.1 and EC. 1.10.3.1) are typically responsible for the formation of eumelanin.

Laccases are multi-copper metalloenzymes that contain four copper ions in their catalytic core. These enzymes have broad classes of substrates, which perform single electron oxidations of hydroxyl groups, mostly attached to aromatic groups (Jones and Solomon 2015; Riva 2006; Yaropolov et al. 1994). In the environment, organisms such as fungi use laccases to oxidize dead material and decompose it; these enzymes are responsible for delignification and decomposition of wood and lacquer formation (Christopher et al. 2014). In the context of melanin production, laccases oxidize the hydroxyl groups on catechols and catecholamines to create unstable phenoxyl radicals. The radicals are unstable and rapidly cause the oxygen to form a double bond with the aromatic ring, forming a quinone. As previously mentioned, the quinone is highly reactive and subject to continued cyclization, oxidation, and polymerization without the need for further enzymatic catalysis. Laccases are important enzymes for melanin production in many fungi, bacteria, and insects. Notably, many disease-causing fungi use laccases to produce melanin,

Fig. 1 Biosynthetic Pathways of Fungal Melanin Production. In fungi, melanin is produced predominately through three pathways: Pyomelanin through the polymerization of homogentisic acid (HGA) produced through the Tyrosine Degradation Pathway, DOPA-melanin through the polymerization of dihydroxyindole (DHI) and dihydroxyindole carboxylic acid (DHICA) produced through tyrosine and L-DOPA oxidation, and the polymerization of 1,8-dihydroxynaphthalene (DHN) produced through the polyketide synthase pathway. "Namesake" molecules are highlighted in yellow. Enzymes responsible or aiding in melanogenesis are bolded. Photograph examples of pyomelanin, courtesy of Dr. Emma Camacho are *Cryptococcus neoformans* (top left) and *C. gattii* (top right) cultures treated with homogentisic acid. Non-pyomelanized controls are seen in the laccase mutant *C. neoformans* (bottom left) and *C. albicans* (bottom right). DOPA-melanin example is H99 strain of *C. neoformans* grown in L-DOPA minimal media. DHN-melanin example is a culture of *Fonsecaea pedrosoi* from (Adibelli et al. 2016) ▶



such as the genus of human pathogenic yeast, *Cryptococcus* spp. (Williamson 1994; Zhu and Williamson 2004). Interestingly, *Cryptococcus* spp. cannot produce melanin from tyrosine and cannot produce their own melanin precursors. They thus require exogeneous catecholamines for laccase substrates (Nurudeen and Ahearn 1979). The fact that *C. neoformans* requires exogenous precursors for the synthesis of melanins means that it is possible to control melanin composition through the substrate provided and this has been exploited to explore the biochemistry of melanin (Chatterjee et al. 2018).

Another class of melanin-producing enzymes is phenol oxidase. Phenol oxidase is a broad umbrella term describing metalloenzymes with two copper ions in the catalytic core, a generally conserved active site, and is able to oxidize phenols and catechols into quinones (Casella et al. 1996; Gerdemann et al. 2002; Monzani et al. 1998; Réglier et al. 1990). Phenol oxidases are divided into two sub-classes of enzymes: tyrosinases and catechol oxidases. Tyrosinases have two distinct enzymatic roles, whereas catechol oxidases only have one of these enzymatic functions. First, tyrosinases have a monooxygenase/monophenolase role, in which a monophenol such as tyrosine is hydroxylated to form an -*o*-diphenol, typically a catechol such as L-DOPA. The second enzymatic activity of the tyrosinase (and the only enzymatic activity of the catechol oxidases) is a diphenolase activity (Ramsden and Riley 2014). This reaction oxidizes catechols such as DOPA into *o*-quinones such as dopaquinone. Similar to the laccase-mediated reaction, the quinone formed from tyrosinase activity is highly reactive and prone to rapid and spontaneous cyclization, oxidation, and polymerization into eumelanin as per the canonical Raper-Mason pathway. Some organisms have additional enzymes to assist in the progression of melanin production post-tyrosinase. These enzymes such as dopachrome tautomerase (Dct) and tyrosinase-related protein 1 (Trp1) help catalyze the process of melanin formation. Dct catalyzes the conversion of the melanin intermediate dopachrome into DHICA, resulting in increased formation of DHICA-based eumelanin compared to the normally more abundant DHI-based eumelanin (Tsukamoto et al. 1992). In mice but not humans, Trp1 oxidizes DHICA (Boissy et al. 1998). Fungi, protozoa, insects, mammals, and birds commonly have tyrosinases, while plants typically have catechol oxidases, sometimes called polyphenol oxidases.

1.2 Characteristics and Synthesis of DHN Melanin

DHN melanin is common among many fungal species including the conidia spores of many filamentous fungi including some *Aspergillus* spp. and *Lomentaspora prolificans* (Al-Laaeiby et al. 2016; Langfelder et al. 1998; Pal et al. 2014; Tsai et al. 1999). DHN melanin arises from the polyketide (pentaketide) synthesis pathway (Fig. 1). Typically, five molecules of the precursor acetyl-CoA or malonyl-CoA are joined together by polyketide synthase enzymes (EC 2.3.1.233) to form a two-ringed molecule called 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) (Austin et al. 2004; Izumikawa et al. 2003; Takano et al. 1995). Subsequently, the molecules undergo two enzymatic reduction and two enzymatic dehydration steps. 1,3,6,8-THN is reduced through a THN reductase (EC 1.1.1.252) into scytalone (Thompson et al. 2000; Wang and Breuil 2002). The scytalone is dehydrated by a scytalone dehydratase (EC 4.2.1.94) to form 1,3,8-THN (Kubo et al. 1983, 1996), which is further reduced by THN reductase into vermelone (Wang and Breuil 2002). This vermelone can be enzymatically dehydrated into 1,8-DHN through scytalone dehydratase (Basarab et al. 1999). The 1,8-DHN is then oxidized to form

polymers of DHN melanin. Some species have laccases that are able to catalyze the polymerization of 1,8-DHN (Sapmak et al. 2015; Sugareva et al. 2006).

The process of DHN melanin formation differs from DOPA melanin production is several ways. One such way is the heavy reliance on enzymes to catalyze the reactions and polymerizations. DOPA melanin synthesis is typically dependent upon one enzyme and predominately occurs through spontaneous and continuous oxidation-reduction cycling. DHN melanin uses an enzyme to essentially synthesize each intermediate. If activity of one of these enzymes is blocked, the intermediate accumulates or spontaneously oxidizes to form different metabolites or pigments (Lee et al. 2003), which is a feature that also makes DHN melanin synthesis able to be inhibited at several different synthesis pathway steps (Wheeler and Klich 1995). An additional difference is that DOPA melanin may at times require exogenous addition of the melanin precursor, as is the case in *C. neoformans* melanization, whereas DHN melanin is produced predominately through abundant metabolites in the cell such as acetyl-CoA and could occur in non-supplemented conditions. Like all melanins, DHN melanin has a dark black color, is acid resistant, and has a stable radical structure.

Many filamentous fungi, such as *Aspergillus* spp. complex produce non-melanin green, yellow, and brown pigments, through polyketide synthesis pathways similar to the pathway that produces DHN melanin, while perhaps not producing DHN melanin at all, like *A. nidulans* (Mayorga and Timberlake 1992). The synthesis of these additional pigments may use the same enzymes, similar precursors, and even produce intermediate products that overlap with the synthetic pathway of DHN melanin (Cary et al. 2014; Wheeler et al. 2008). For instance, *T. marneffei* uses the PKS pathway to produce several pigments and mycotoxins besides DHN melanin (Tam et al. 2015), and *Aspergillus* spp. that use PKS for a myriad of products as reviewed in (Bhetariya et al. 2011). The non-melanin pigments synthesized by PKS may result in the mistaken understanding that a fungus only known to produce DOPA melanin—such as *A. nidulans*—is actually a DHN-melaninized fungus. Some articles base the existence of DHN melanin merely on PKS genes present. This can lead to discrepancies in literature as to what type(s) of melanin(s) is present in a fungus. There are reports that *A. niger* produces DHN melanin through the PKS pathway, while other reports indicate that *A. niger* exclusively produces DOPA melanin (Chiang et al. 2011; Pal et al. 2014). That being said, we did our best in trying to sort out which melanins are produced by each fungus in Table 1, and we recognize that there could be future discrepancies as more biochemical and biophysical studies are done on the precise nature of the melanins.

1.3 Characteristics and Synthesis of Pyomelanin

Pyomelanin, also known as homogentisic acid (HGA) melanin, is a light-brown pigment that is produced by some bacteria including *Pseudomonas* spp. (Serre et al. 1999; Yabuuchi and Ohyama 1972), and fungi including *Aspergillus fumigatus,*

Histoplasma capsulatum, Sporothrix schenckii, and *Penicillium* (Almeida-Paes et al. 2012, 2018; Schmaler-Ripcke et al. 2009; Vasanthakumar et al. 2015). Similar to many DOPA melanins, pyomelanin may be derived from tyrosine, specifically the degradation pathway of tyrosine (Turick et al. 2010). Tyrosine is deanimated through an aminotransferase to form 4-hydroxyphenylpyruvate (4-HPP) (EC 2.6.1.5). 4-HPP is converted into homogentisic acid by a dioxygenase enzyme (EC 1.13.11.27) (Singh et al. 2018). Homogentisic acid spontaneously oxidizes into benzoquinone acetate (Schmaler-Ripcke et al. 2009), which polymerizes to form pyomelanin (Fig. 1). Alternatively, pyomelanin can be produced directly from the oxidation of HGA, as seen in *C. neoformans* which produces a pigment when it is grown with homogentisic acid. This is presumably pyomelanin produced via direct laccase oxidation (Frases et al. 2007). Pyomelanin is acid resistant, however, unlike DOPA melanin or DHN melanin, it is highly soluble in water. Pyomelanin was first described in *P. aeruginosa* as a water-soluble brown pigment (Yabuuchi and Ohyama 1972).

2 Melanin Localization, Structure, and Remodeling

Melanin and melanin intermediates appear toxic and may induce oxidative stress for cells through production of free radicals, a property of melanin that insects use to their benefit in their innate immune response. By encapsulating pathogens with melanin, insects are able to use the oxidative stress to kill the invader (Graham et al. 1978; Zhao et al. 2011). The cytotoxicity of melanin is one of the reasons that melanin localization tends to be highly organized and compartmentalized throughout kingdoms of life. In mammals, melanin and melanogenesis are confined to a membrane-bound organelle called a melanosome, which it is hypothesized to help to retain the toxic compounds and prevents oxidative stress (Denat et al. 2014). There is evidence that fungi also have membrane-bound melanosomes within their cytoplasm. Intracellular melanin-containing organelles have been reported in *Fonsecaea pedrosoi*, a mold responsible for chromoblastomycosis in humans (Franzen et al. 2008). On an ultrastructural level, these organelles are similar to mammalian melanosomes. Additionally, fungal melanosomes have recently been reported in *C. neoformans* (Camacho et al. 2019). The cryptococcal melanosomes described here are produced in the cytoplasm and exported to the cell wall inside of vesicles and multivesicular bodies (MVBs), where they form dense concentric shells of deposited melanin, and correlate to duration of cell melanization (Eisenman et al. 2005) (Fig. 2b).

This extracellular localization of melanin is common throughout the fungal kingdom, as many melanotic fungi deposit melanin in or around their cell walls, a reaction thought to be mediated by binding between melanin and the cell wall chitin and chitosan or another scaffolding molecule. In *C. neoformans,* mutants that lack functional chitin synthase 3 (*CHS3*) exhibit a "leaky" melanin phenotype (Walton et al. 2005). This phenotype is when melanin leaks from *C. neoformans* colonies, leaving the media pigmented and the colonies and cells white. *CHS3* is necessary

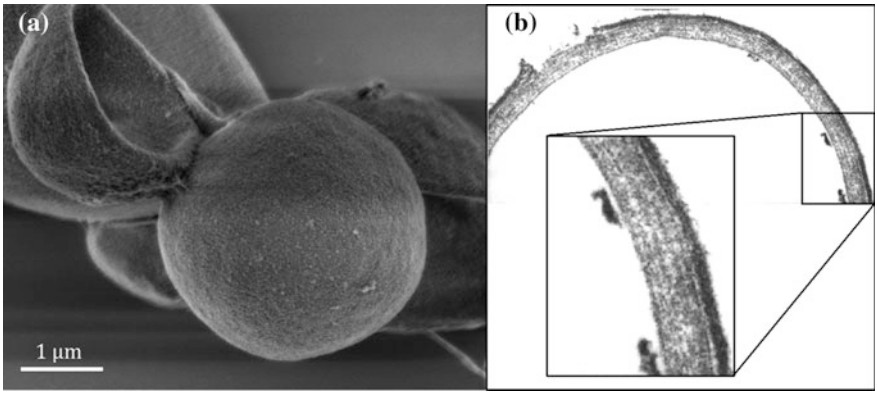


Fig. 2 Structure of Melanin in *Cryptococcus neoformans*. **a** An SEM image of a *C. neoformans* "melanin ghost" from a culture grown with L-DOPA. Image is courtesy of Dr. Emma Camacho. Scale bar indicates 1 µM. **b** A TEM of a melanin ghost from *C. neoformans* with an inset showing the concentric rings of melanin granules formed in the cryptococcal cell wall. Image courtesy of Dr. Helene Eisenman

for proper cell wall integrity, normal growth, chitin production, and chitosan production (Baker et al. 2007; Banks et al. 2005). The inability of *CHS3* mutants to retain their melanin is consistent with the notion that melanin localization and adherence to the cell wall is dependent upon properly formed chitin and chitosan cell wall structures. Similarly, dyes that bind to the cell wall also prevent melanin deposition, resulting in a phenotype similar to that of the leaky mutants, in which melanin is present in high levels in the media (Perez-Dulzaides et al. 2018). Melanin has been reported in the cell walls of *Candida albicans* cultures grown with L-DOPA, as well as during in vivo *Candida* infections (Morris-Jones et al. 2005). When the substrate for chitin synthesis, N-acetylglucosamine, is added to the media, melanin deposition in the cell wall increases (Walker et al. 2010). Conversely, when chitin synthase genes were knocked out, there were major changes in *C. albicans* melanin deposition in the cell wall and externalization (Walker et al. 2010). The *CSH2* mutant did not secrete melanin into the extracellular space, and melanin remained within the cell. Other chitin synthase mutants, such as the *CSH3* mutant, secreted "melanosomes" that remained embedded in the cell wall rather than fully secreted into extracellular space.

Externalization of melanin could provide an extracellular physical and chemical shield against stressors or factors within the host or environment. The external layers of filamentous fungal conidia cell walls are often melanized, such as *Aspergillus* spp., *L. prolifcans, F. pedrosoi.* This external localization of conidial melanin could help prevent physical, chemical, and biological degradation (Gessler et al. 2014), as well as prevent the desiccation of the spore and promote survival in harsh conditions (Tudor et al. 2012).

While the cell wall localization of fungal melanin is practical as per its function, it also provides a logistical issue for the fungi as it grows and divides. Melanized *C.*

neoformans must be able to remodel its thick layer of cell wall melanin before undergoing budding in order to allow mitosis and cytoplasmic contents to be conferred to the daughter cell. Melanin remodeling is not completely understood after decades of investigation. However, it is theorized to be a process mediated by reconfiguration of melanin granules in the cell wall, which have been described as small 50–80 nm (Eisenman et al. 2005) up to 200 nm (Camacho et al. 2019) spherical units of melanin associated with the cell wall. These larger melanin granules are in turn composed of smaller "fungal melanosomes," roughly 30 nm in diameter (Camacho et al. 2019). Through electron microscopy and atomic force microscopy, the "melanin ghost"—a melanin shell remaining intact after the rest of cell material is enzymatically and chemically decomposed (Fig. 2a)—of *C. neoformans* appears to be composed of concentric layers of these melanin granules (Fig. 2b, c) (Eisenman et al. 2005). These layers are presumably added as the cell ages, with the innermost portion of the cell wall melanizing first followed by the more external cell wall regions. Newly formed daughter buds have fewer of these concentric layers of melanin (Eisenman et al. 2005). The melanin granules are tightly associated but become dissociated with increased duration of acid hydrolysis, suggesting that they are separate units tethered together to form a "bead on a string" structure (Camacho et al. 2019; Eisenman et al. 2005). These beaded string structures have also been reported for melanin granules in the cell wall of *P. marneffei* (Liu et al. 2014). This provides a potential mechanism by which cryptococcal melanin is remodeled to allow for cell division and cell growth, where the individual melanin granules are dynamic and rearranged and restructured at will, akin to rearranging bricks in a wall to create a passageway.

In recent years, *C. neoformans* was found to secrete extracellular vesicles or exosomes. These are membrane-bound structures up to 100 nm in diameter that are somehow able to be secreted past the cell wall and capsule (Wolf et al. 2014). Although the capsule was thought to be a rigid structure that would not allow transport of such large structures as exosomes, recent evidence shows that its viscoelastic properties permit vesicular transit (Walker et al. 2018). These exosomes contain a wide variety of virulence factors such as urease and secreted polysaccharide (Rodrigues et al. 2008). For this reason, the cryptococcal extracellular vesicles are often termed "virulence bags". In addition to these other virulence factors, the exosomes contain laccase, the enzyme necessary for *Cryptococcus* to produce melanin. In some instances, the exosomes appear to contain melanin pigment (Rodrigues et al. 2008). Additionally, preparations of exosomes have laccase enzymatic activity (Rodrigues et al. 2008). This has implications for *Cryptococcus* pathogenesis, as these secreted vesicles are capable of making melanin and are theoretically able to disseminate throughout the host in concentrated packages. Throughout the body and in the area of infections, these exosomes can have impact on the host via the same properties of cell-bound melanin. Given that all microbes that have been investigated produce exosomes (Deatherage and Cookson 2012), the propensity for virulence factors to be packed inside exosomes (Schorey et al. 2015), and the common trend of melanin as a fungal virulence factor (Polak 1990), it stands to reason that melanin and melanin-producing enzymes may

be common in fungal exosomes. Additionally, it is possible that melanotic exosomes can play a significant role in microbial pathogenesis through interaction with the host, including eliciting an anti-melanin antibody response or acting as a sponge for microbicidal agents.

3 Roles of Melanin in Fungal Pathogenesis

Melanin is commonly found in many fungi, indicative of its important role in fungal survival. Many—if not all—human pathogenic fungi produce melanins. The melanization of fungal pathogens is diverse and includes DOPA melanins, DHN melanins, and pyomelanins. Fungal melanin characteristics and its relation to pathogenesis are summarized in Tables 1 and 2, respectively, which includes common human pathogens such as *Cryptococcus* spp., *Aspergillus* spp., *Candida albicans, Sporothrix schenckii,* and *Histoplasma capsulatum.*

There is often a direct association between degree of melanization and the virulence of fungal pathogens. For example, a mutant strain of *C. neoformans* with a deletion in the region of *CNLAC1*, the primary cryptococcal gene that encodes laccase, is less virulent in both mouse and *Galleria mellonella* infection models (Zhu and Williamson 2004). Further, when a melanin inhibitor is used to treat mice infected with a wild-type strain of *C. neoformans*, they have prolonged survival compared to the survival of control-treated groups. This is seen in the case of treatment of infected mice with glyphosate, a melanin inhibitor in fungi (Nosanchuk et al. 2001). Similarly, *E. dermatitidis* strains that did not produce melanin were less pathogenic than normally melanized strains (Dixon et al. 1992). Clinical isolates of *Sporothrix* spp. associated with higher melanin content were also found to be more virulent, with more dissemination to internal organs (Almeida-Paes et al. 2015).

3.1 Oxidative Stress Protection

One of the most prominent uses of melanin in biology is based on its role as an antioxidant and shield against free radicals from fungi to mammalian retina and skin (Brenner and Hearing 2008; Wang et al. 2006). While melanin and its intermediates are often toxic and highly oxidative due to the free radicals they produce, it is simultaneously well established to be a strong antioxidant with a stable free radical structure that is able to neutralize other free radicals (Jacobson and Tinnell 1993; Różanowska et al. 1999). Melanin is able to quench free radicals and reduce the oxidative stressors of the environment around it. This could be useful in the environment to help microbes manage oxidative stress (Gessler et al. 2007; Jacobson and Tinnell 1993).

Table 2 Roles of fungal melanins in pathogenesis

Species	Disease	Virulence	Oxidation protection	Antifungal resistance	AMP resistance	Enzyme inhibition	Antibody response	Effect on phagocytosis	References
Aspergillus fumigatus	Aspergillosis	✓	✓	✓	ND	ND	✓	+	Jahn et al. (1997), van de Sande et al. (2007), Volling et al. (2011), Youngchim et al. (2004)
Aspergillus nidulans	Aspergillosis	ND	✓	ND	ND	✓	ND	ND	Gonçalves and Pombeiro-Sponchiado (2005), Kuo and Alexander (1967)
Candida albicans	Candidiasis	✖	ND	ND	ND	ND	ND	ND	Walker et al. (2010)
Cryptococcus neoformans	Cryptococcosis	✓	✓	✓	✓	✓	✓	–	Doering et al. (1999), Duin et al. (2002), Nosanchuk et al. (1998), Rosas and Casadevall (2001), Wang et al. (1995)
Exophiala dermatitidis	Phaeohyphomycosis	✓	✓	✓	✓	✓	ND	None	Paolo et al. (2006), Schnitzler et al. (1999)
Fonsecaea pedrosoi	Chromoblastomycosis	✓	✓	ND	ND	ND	✓	+ or −	Alviano et al. (2004), Cunha et al. (2005), Cunha et al. (2010)
Histoplasma capsulatum	Histoplasmosis	ND	ND	✓	ND	ND	✓	ND	Duin et al. (2002), Nosanchuk et al. (2002)
Lomentospora prolificans	Lomentosporiosis	ND	✓	✖	ND	ND	ND	ND	Al-Laaeiby et al. (2016)
Paracoccidioides brasiliensis	Paracoccidioidomycosis	✓	✓	✓	ND	ND	✓	–	da Silva et al. (2006), Urán et al. (2011)

(continued)

Table 2 (continued)

Species	Disease	Virulence	Oxidation protection	Antifungal resistance	AMP resistance	Enzyme inhibition	Antibody response	Effect on phagocytosis	References
Sporothrix schenckii	Sporotrichosis	✓	✓	✓	ND	ND	✓	–	Almeida-Paes et al. (2016), Morris-Jones et al. (2003), Romero-Martinez et al. (2000)
Talaromyces marneffei	Penicilliosis	✓	✓	✓	ND	ND	✓	–	Liu et al. (2014), Kaewmalakul et al. (2014), Woo et al. (2010), Youngchim et al. (2005)

Key: Fungus' melanin is does (✓) or does not (✖) play role, *ND* indicate not determined to play role, + indicates increased phagocytosis, - indicates decreased phagocytosis.
Abbreviations Antimicrobial peptide (AMP)

This property of melanin is also useful within host tissues upon infection and encountering the oxidative defenses of the immune system. When a microbe is recognized by the host defenses, it is targeted by oxidative stress produced by the innate immune system. Macrophages, monocytes, and neutrophils release reactive oxygen species (ROS) including superoxide and peroxide ions, as well as nitric oxide (Babior 1978; Marcinkiewicz 1997; Nathan and Hibbs 1991). These chemicals are highly cytotoxic and are typically effective in killing foreign cells, however, melanin is capable of neutralizing the oxidative bombardment. As a result, many melanized fungal pathogens are resistant to oxidative/respiratory bursts produced by the innate immune system, including *C. neoformans* (Wang and Casadevall 1994) and *A. nidulans* (Ruiz-Díez and Martínez-Suárez 2003). The oxidative respiratory bombardment continues within the phagosome (Bedard and Krause 2007; Kotsias et al. 2012; Winterbourn et al. 2016). The antioxidant property of melanin, along with other virulence factors such as urease, may allow the melanized cell to survive once engulfed and residing within the phagosome.

While melanin production creates superoxide molecules (Komarov et al. 2005), formed melanins are able to quench and detoxify superoxide ions (Tada et al. 2010). This signifies that melanin has some redundant roles with superoxide dismutase (SOD), a common class of antioxidant enzymes and known virulence factors in *C. neoformans* and *C. albicans* (Hwang et al. 2002; Jacobson et al. 1994). Melanin and SOD can work together in microbial pathogens in order to evade host-produced oxidative bursts, as they may not be localized together—melanin is often extracellular and located in the cell wall of fungi, and SOD is often intracellular within in the mitochondria or cytosol (Broxton and Culotta 2016), although some species of fungi have SOD reported to presented on the cell surface or within extracellular vesicles to some degree (Gleason et al. 2014; Rodrigues et al. 2008; Youseff et al. 2012).

One interesting potential use of this redundancy is in instances of high-temperature environments. Under such conditions, the melanin is heat stable and continues to quench superoxide and oxygen radicals, while the SOD's enzymatic activity is diminished and unable to perform its protective functions at high temperatures. Conversely, in *C. neoformans,* levels of melanization are reported to be lower at cultures grown at higher temperatures, whereas levels of SOD are higher at 37 °C than at lower temperatures (Jacobson et al. 1994). This inverse relationship implies a possible compensatory mechanism. Similarly, but outside of the fungal kingdom, melanin and SOD have been reported to have complementary and compensatory roles in reducing oxidative stress in amphibian livers. In the liver of amphibians, there is an inverse relationship between melanin and SOD expression (Schiel et al. 1987). The lower the melanin content of the liver is, the more SOD is expressed. High melanin can quench a majority of the superoxide ions and cause the low SOD levels effect due to a feedback loop between superoxide concentration and SOD expression.

3.2 Antifungal Drug Sequestration

Melanin is able to sequester some antifungal drugs. The external localization of the melanin is important for this role in virulence; being located in the cell wall keeps antifungal compounds in extracellular space and thus prevents the drugs' cytotoxic effects. In *C. neoformans* and *H. capsulatum,* melanin is able to absorb amphotericin B, an antifungal commonly used in clinic, and caspofungin, the original drug from the commonly used echinocandin class of antifungals (van Duin et al. 2002). The presence of melanin and L-DOPA in the media allows the fungal cells to survive better following amphotericin treatment, and it was found that isolated melanin from *C. neoformans* was able to successfully bind amphotericin B in media. Following conditioning with melanin, this amphotericin-containing media showed an increased minimum inhibitory concentration (MIC) when used to grow *C. neoformans* cultures compared to the non-conditioned control media. This indicates the antifungal potency of the media was lost, likely due to absorption and binding of melanin to amphotericin B. Similar observations that melanin protects fungi from antifungals have been reported in *Paracoccidiodes brasiliensis*, where melanin did not effect the MIC of the drugs tested, but a smaller percentage of melanized cells were killed compared to the percentage of non-melanized cells killed by amphotericin B, ketoconazole, fluconazole, itraconazole, and sulfamethoxazole (da Silva et al. 2006). Media containing the antifungal azoles itraconazole and ketoconazole were conditioned with isolated melanin from *Madurella mycetomatis*. The melanin conditioning was found to increase the MIC (van de Sande et al. 2007). Using tricyclazole, a DHN melanin inhibitor, makes the fungi *S. brasiliensis* and *S. schenckii* more susceptible to killing by the antifungal drug terbinafine, whereas inhibition of eumelanin and supplementation with L-DOPA do not seem to affect susceptibility or MIC of terbinafine (Almeida-Paes et al. 2016). The DHN melanin-producing fungi *Alternaria infectoria* increased the production of melanin in response to certain antifungal drugs, notably, nikkomycin Z, caspofungin, and itraconazole treatment all increased melanin production (Fernandes et al. 2016).

Importantly, melanin does not bind all drugs, and not all fungal melanin contributes to drug resistance. It has been reported in *C. neoformans*, *H. capsulatum,* and *M. mycetomatis* and several other black fungi that their melanin does not confer protection from or bind to fluconazole (van Duin et al. 2002; van de Sande et al. 2007), which is a popular member of the azole class of antifungals. However, melanized *P. brasiliensis* is less susceptible to fluconazole as well as other azoles such as itraconazole, ketoconazole, and sulfamethoxazole (da Silva et al. 2006). Melanin-conditioned media using isolated melanin from *M. mycetomatis* did not show altered MIC for amphotericin B, suggesting that the *M. mycetomatis* melanin does not bind this drug as other fungal melanins do (van de Sande et al. 2007). Additionally, melanin has not been shown to play a role in the high amphotericin B resistance of *L. prolificans* (Al-Laaeiby et al. 2016; Ruiz-Díez and Martínez-Suárez 2003). In the more recent study, polyketide synthase (*PKS*) and scytalone

dehydratase (*SCD*), two genes necessary for the DHN melanin production pathway were knocked out, and the mutants were analyzed for susceptibility to antifungals. It was found that the albino mutants were not susceptible to amphotericin compared to the melanized wild type.

Knowledge of which drugs get absorbed by melanin is medically important, as it represents a way clinicians could treat melanized fungi without having to deal with the pharmacoevasive effect of pigment.

As of this writing, melanin's protective effect in live fungi and its in vitro mechanism of antifungal drug sequestration is unknown. It is possible that the melanin is directly binding these antifungal drugs and thereby sequestering them, which is supported by the apparent absorption of the drugs by isolated melanin ghosts. An additional possibility is that the melanin is interfering with the oxidative burst that these antifungals, namely amphotericin B, may induce in order to kill the fungi (Mesa-Arango et al. 2014).

3.3 Protein Binding

Melanins have been shown to bind peptides strongly. One indication of this is that isolated melanin ghosts from *C. neoformans* still have proteins associated with them via NMR analysis, and "ghosts" of melanin granules isolated from *C. neoformans* still have peptide fragments that are identifiable through mass spectrometry (Camacho et al. 2019). These peptides fragments are so tightly bound and protected by melanin that they remain in the melanin ghosts after extended acid hydrolysis, enzymatic treatment, and lipid extractions involved in the production of the melanin ghosts (Wang et al. 1996). This allows for interesting insight in understanding which proteins that peptides interact with melanin during fungal melanogenesis. Other studies indicate that melanin non-specifically binds proteins from both mammalian serum and fungal lysates (Doering et al. 1999). The protein binding was dependent upon pH—the more acidic conditions resulted in less protein binding to the melanin. This finding indicates that melanin–protein binding might be due to binds to electrostatic interactions. Melanin is a negatively charged pigment, so the protons present in acidic environments might neutralize the melanin and prevent the attraction and binding to positively charged proteins.

In the context of infection and pathogenesis, the peptide-binding role of melanin could be important. Melanins derived from *C. neoformans* were found to inactivate or absorb antimicrobial peptides produced by the host immune system, such as defensins, protegrins, and magainins (Doering et al. 1999). The media containing the antimicrobial peptides was less effective at preventing fungal growth when treated with melanin versus untreated. This represents a way in which melanin blocks the microbicidal actions of the host immune system.

Melanin was also reported as providing a line of defense against enzyme-dependent degradation and killing. There have been associations between melanization and resistance to chitinases and glucanases for decades, primarily in